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CFZ is not inducing ER stress in HUVECs

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Student's Name

Institution Affiliation

Course

Professor

Date

CFZ is not inducing ER stress in HUVECs

3.1 Introduction

Multiple myeloma (MM) is a malignant transformation of plasma cells that collect in bone marrow, leading to marrow failure and bone destruction(1). The disease is more frequent in the oldest population, and it counts double in blacks' people frequently as whites' people(2). The mean age of patients with MM is 61 years for women (79% > 70 years) and 62 years for men (75% > 70 years). The root of this disease is not fully understood yet, and its extent, clinical course, sensitivity to drugs, and complications differ widely among patients(2).

MM treatment has improved noticeably in the past decade. The expansion in the number of active drugs has created several potential drug combinations that can be used in the first line and relapsed settings(3). In addition, a greater understanding of the cellular disease mechanisms emerged as a foundation for new combinations of treatments and new drug development(4). According to the Surveillance Epidemiology and End Results database, there was a significant increase in the five-year survival rate between 1975 (25%) – 2005 (34%) due to the administration of advance and more effective treatment regimens(5).

Even though MM is usually sensitive to numerous cytotoxic treatments, both as primary treatment and as management of relapsed disease, responses to the drugs are transient; thus, MM final cure is extremely challengeable with existing approaches(4). The current drawbacks of MM treatment approaches lead to disease relapse and refractory to all available treatments. Therefore, developing a new, more effective, and a well-tolerated class of MM drugs was essential (6).

The 26S proteasome plays a vital function in cellular homeostasis throughout its role in ubiquitin-dependent protein turnover, involving targets misfolded and obsolete proteins, stress

response, apoptosis, cell-cycle progression, and DNA repair(7). Thus, the proteasome has emerged as a vital objective for cancer treatment with the authorisation of bortezomib (BFZ), a first-class reversible proteasome inhibitor (PIs) for (RRMM)(6). Nevertheless, BFZ was ineffective among some MM patients and others who developed drug resistance, which was an essential trigger to establish other PIs with superior activity. CFZ is a novel, evaluated irreversible epoxomicin-related second-generation PIs. This agent effectively bound and exclusively inhibited the chymotrypsin-like immunoproteasome and proteasome activities(8). This inhibition leads to the accumulation of ubiquitinated substrates within the cellular cytosol, thus ER stress and activation of unfolded protein response system(8).

Prolonged activation of this system and failure of the cells to restore normal protein production ultimately led to apoptosis. Programmed cell death was associated with mitochondrial membrane depolarization, activation of both intrinsic and extrinsic caspase pathways, activation of c-Jun-N-terminal kinase, and release of cytochrome *c*(8). Carfilzomib has significantly advanced the management of relapsed and/or refractory MM patients, involving those intolerant to bortezomib(6).

Great response rates have been confirmed with carfilzomib as a single treatment or combined with corticosteroids, immunomodulators, and alkylating agents, including patients who have failed various prior treatments(6). Carfilzomib also has substantial potential in the MM frontline treatment approach, supporting response and survival rates(6). Importantly, carfilzomib presented improved effectiveness compared with bortezomib showed effectiveness against bortezomib-resistance clinical samples and bortezomib-resistant MM cell lines(8). Carfilzomib also overwhelmed resistance to other standard treatments and operated synergistically with dexamethasone to increase cancer cell death(8). However, the usage of CFZ in MM has been associated with an increased risk of serious adverse events. Various cardiovascular complications were reported among these events, including hypertension,

myocardial infarction, ischemic heart disease, angina, and others(9). Among 60 MM patients treated with carfilzomib, 12% (95% confidence interval, 3.8%-20%) of them were estimated for cardiovascular risk complications(10). They experienced ≥20% reversible left ventricular ejection fraction (LVEF), a principal indicator of cardiac dysfunction. The incidence of LVEF reduction was 5% at three months, 8% at six months, 10% at 12 months, and 12% at 15 months(10).

In this result chapter, we hypothesised that the cytotoxic effect of CFZ against MM cancer cells could be an off-target effect against the human vascular endothelium. Therefore, we investigated the potential ability of CFZ to inhibit the proteasome function of the endothelium, thus activation of ER stress in the endothelium.

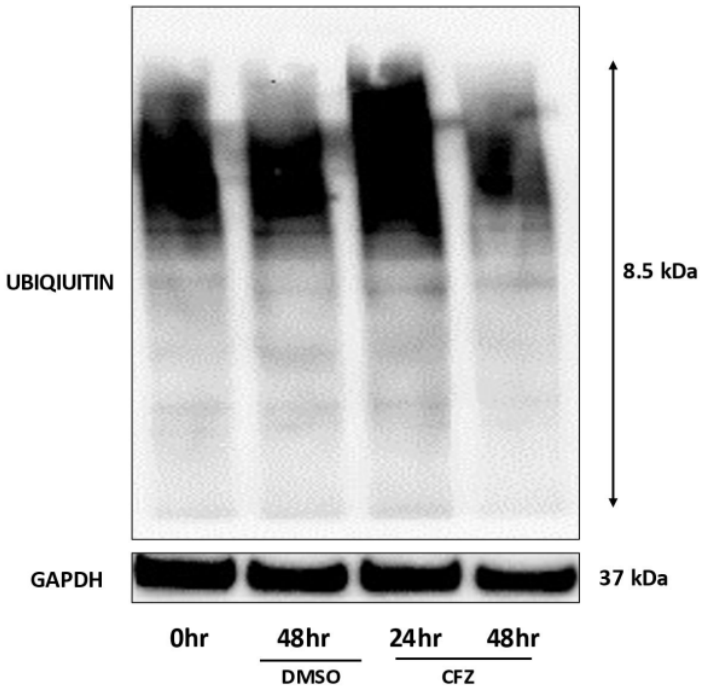
3.2 Results

3.2.1 carfilzomib induces protein ubiquitination and morphological changes in HUVECs in vitro

Ubiquitin is a protein implicated in the regulation of misfolded protein degradation through ERAD mechanisms. The build-up of ubiquitination chains triggers the ERAD by the proteasome. In order to investigate our initial hypothesis that CFZ alters and damage endothelium function in a similar approach as its effect against multiple myeloma cancer cells by inducing an ER stress, thus cellular failure to maintain normal protein production, which terminates to initiate apoptosis. In clinical practice, CFZ is given in the concentration range 500-3000nM, and in vivo, the pharmacokinetics half-life of the drug is 1 hr. Initially, we investigated if CFZ induces proteasome inhibition of endothelial cells; HUVECs were treated with 750nM of CFZ for 1hour, followed by removal of the drug and replacement with new media. At selected time-points (0hr,24hr, and 48hr) during recovery, the cells were harvested, and cell lysates were collected. Western blot was used to compare the ubiquitination expression

level of CFZ treated cells compared to non-treated, and solvent DMSO controls. The immunoblotting results obtained across three biological repeats indicated that ubiquitination was significantly up-regulated in CFZ-treated cells after 24 hr compared to the controls, which is consistent with the role of the drug as a proteasome inhibitor (**Figure.1A&B**). In concordance, Light microscopy reveals a progressive morphological change "stretched" in CFZ treated cells up to 24 hr recovery (**Figure.1C-C**), indicating structural rearrangement or activation of the endothelium; while in 48 hr recovery, the cells were almost returned to a similar appearance as untreated and DMSO normal "cobblestone "cell morphology, indicating cell recovery (**Figure.1C-A, B, D**). Taken all these observations together, CFZ has an evident potent ability to inhibit the proteasome function in the endothelium; however, this was a transit effect since the ubiquitination level and the cell morphology almost returned to a comparable level and appearance as the controls cell after 24 hr recovery.

A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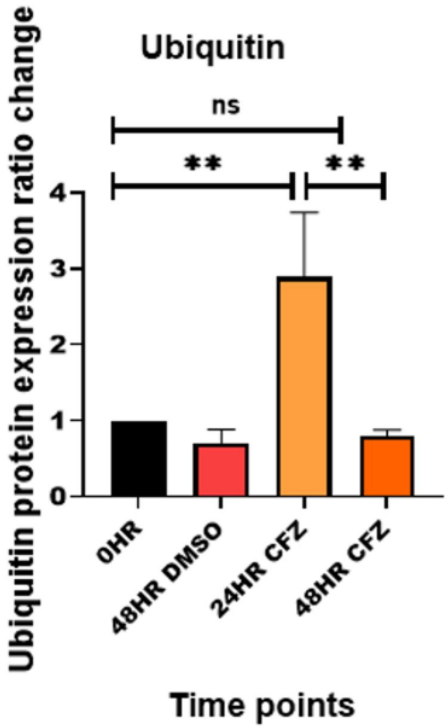
GAPDH
37 kDa
0hr
48hr
24hr
48hr
DMSO
CFZ

B


Ubiquitin
ns
**
**
Ubiquitin protein expression ratio change
0
1
2
3
4
0HR
48HR DMSO
24HR CFZ
48HR CFZ
Time points

N=3

C

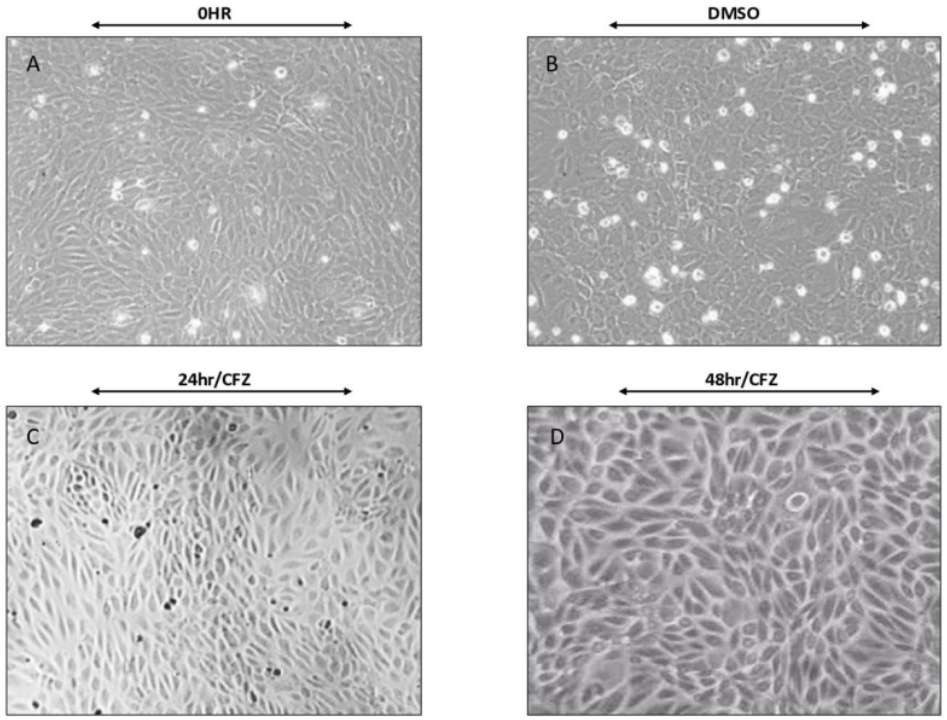
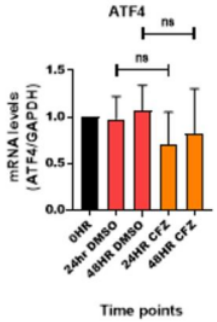


Figure 1: CFZ is inducing proteasome inhibition and morphological change to HUVECs. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. Western blot was used to determine the extent of ubiquitination. ***A***) Western Blots analysis of ubiquitin-protein level expression. **B)** Density quantification of western blot**. C)** Morphological observation using a light microscope (10X lens). DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

3..2.2 Carfilzomib does not induce ER stress in HUVECs

*Following the observation of morphological alteration in HUVECs caused by CFZ, CFZ stimulated ER-stress in HUVECs was examined as a possible core molecular mechanism. To assess if CFZ induces ER stress in endothelial cells, HUVECS were treated with CFZ for one hour as described in (**section 2.2**), cells were left to recover for 24hr and 48hr, followed the cells were collected for gene and protein expression analysis of a panel of known ER stress markers (BIP, PERK, IRE1α, ATF6, CHOP, and ATF4) using qPCR and immunoblotting respectively. Interestingly, the data obtained showed that CFZ did not induce ER stress in HUVECS at any tested time points. qPCR demonstrated similar gene expression profiles for all the genes tested (**Figure.2**). This was furthered confirmed by western blot analysis (**Figure.3, A&B**). These data shows that at least at this dose of CFZ ER stress is not induced in HUVECS.*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ns
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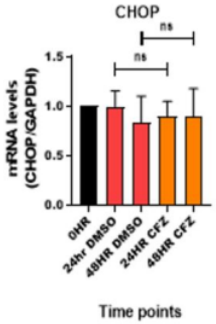
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points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ns
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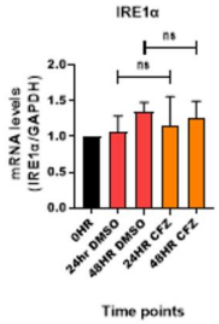
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points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ns
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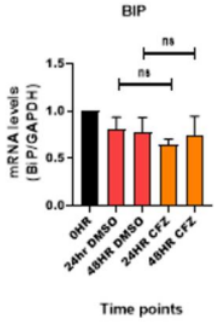
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points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ns
mRNA levels (BiP/GAPDH)
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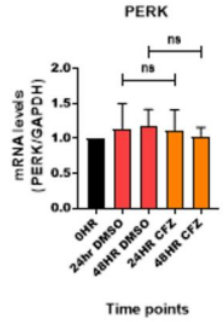
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points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ns
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1.0
0.5
0.0
0HR
24hr DMSO
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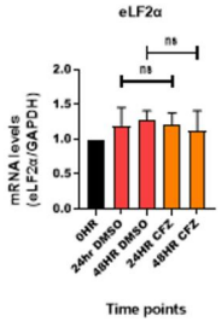
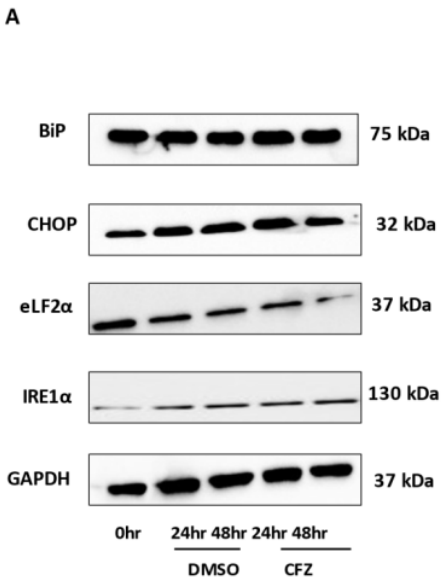
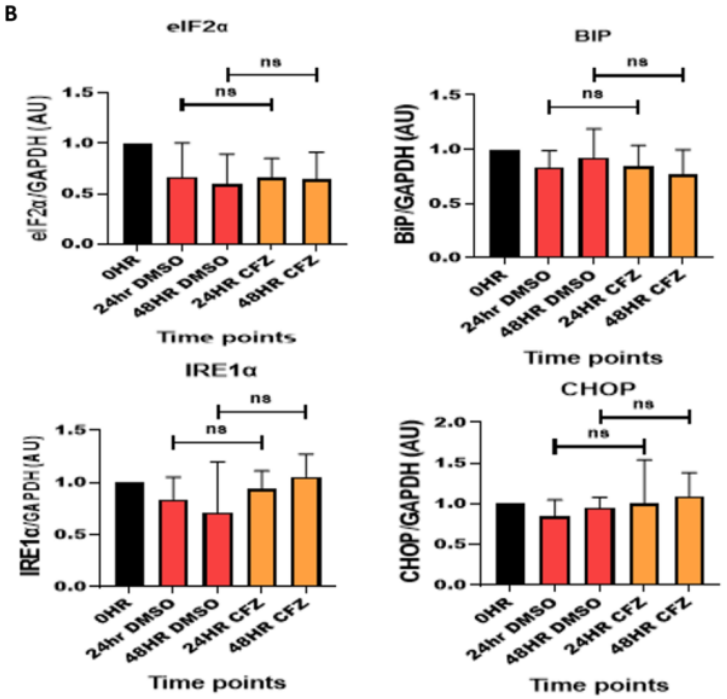
24HR CFZ
48HR CFZ
Time points


eLF2α
ns
ns
mRNA levels (eLF2α/GAPDH)
2.0
1.5
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points

N=3

Figure 2: CFZ is not inducing ER stress to HUVECs. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspending them in a fresh EBM-2 medium. Cells were harvested to extract RNA at 0hr, 24hr, and 48hr. cDNA was prepared from RNA to quantify mRNA expression using q-PCR. The expression level of mRNA of principal's ER stress markers (BIP, PERK, eLF2α, ATF4, CHOP, and IRE1α) in CFZ treated HUVECs and controls. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in each experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represent the average of four independent experiments. The expression trends of mRNA and protein species between CFZ and DMSO cells are compared using one-way ANOVA. Significant: ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.001


A
BiP
75 kDa
CHOP
32 kDa
eLF2α
37 kDa
IRE1α
130 kDa
GAPDH
37 kDa
0hr
24hr 48hr 24hr 48hr
DMSO
CFZ


B
eIF2α
eIF2α/GAPDH (AU)
ns
ns
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points
BIP
BiP/GAPDH (AU)
IRE1α
IRE1α/GAPDH (AU)
CHOP
CHOP/GAPDH (AU)

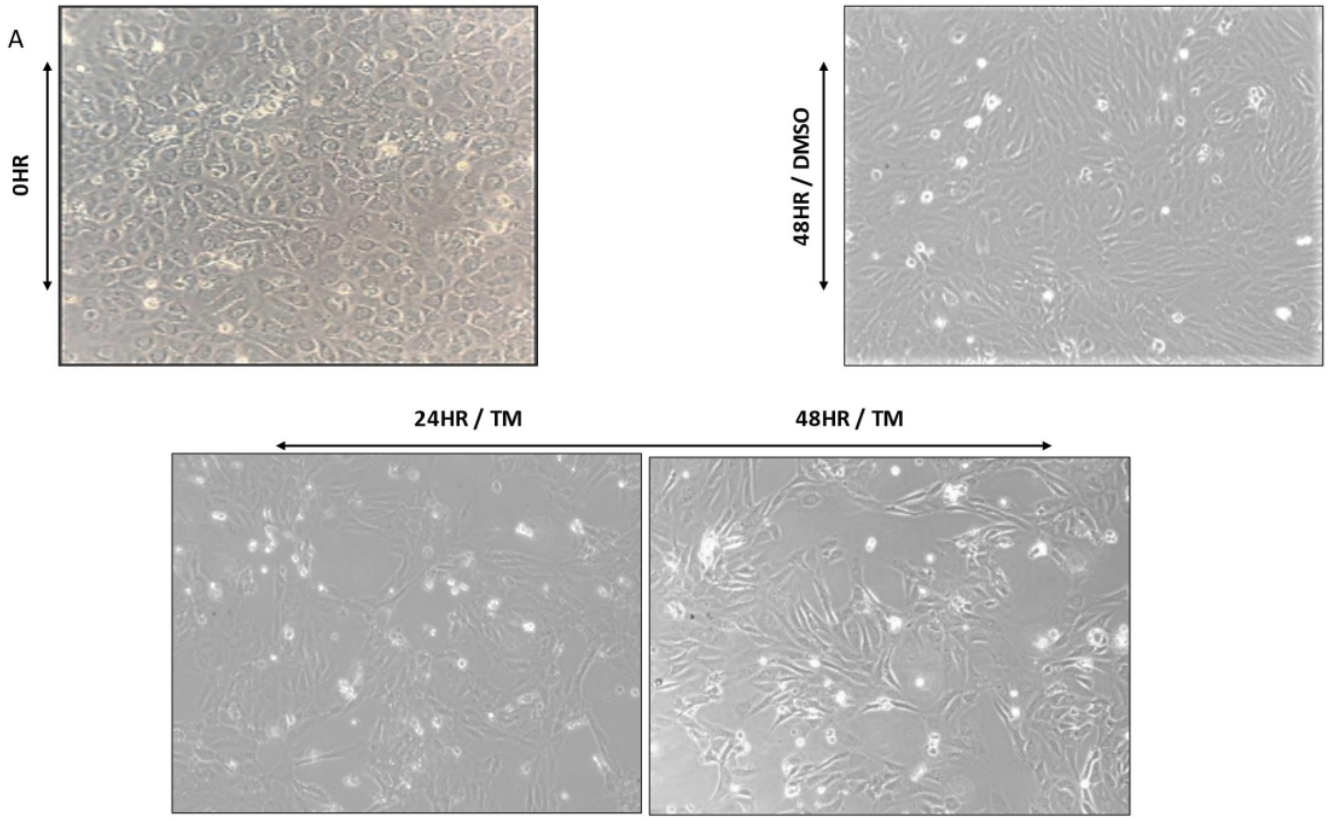
N=3

Figure 3: ER stress protein expression of CFZ treated HUVECs compared to controls. HUVECs were treated with 750nM of CFZ and DMSO for 1hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. Western blot was used to determine the protein level. A) Western Blots bands of BiP (75 kDa), CHOP (32 kDa), elF2α-p (37 kDa), IRE1α-p (130 kDa) and GAPDH (37kDa). B) density quantification of western blots. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in each experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of mRNA and protein species between CFZ and DMSO cells are compared using one-way ANOVA. Significant: ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.00.

3..2.3 Tunicamycin induced ER stress in HUVECs.

Since the data obtained from the former CFZ experiments showed a lack of CFZ ability to induce ER stress in HUVECs under the experimental conditions, the tunicamycin, a well-established ER stress inducer in HUVECs, was selected as an ER stress inducer positive control(11). Tunicamycin (TM), a naturally occurring antibiotic, stimulates ER stress in cells by preventing the biosynthesis of the N-linked glycan, which is considered a first step in the proteins synthesis that subsequent many misfolded proteins accumulate in the cytosol; thus, induction of ER stress(11). It is frequently used *in vitro* to investigate the effects of ER stress(11). To investigate tunicamycin's ability to induce ER stress in our endothelial cells, HUVECs were treated with ten μg/ml of tunicamycin for 24 hours, followed by removal of the drug and replacement with new media. At selected time-points (0hr, 24hr, and 48hr) during recovery, the cells were harvested, and cell lysates were collected. Gene and protein expression analysis of a panel of known ER stress markers (BIP, *PERK*, *IRE1α*, *ATF6*, *CHOP*, and *ATF4*) was monitored using qPCR and immunoblotting, respectively.

Interestingly, tunicamycin induced ER stress in HUVECs at all the tested time points. In addition, there was clear morphological change between TM group and the controls. The cells were extremely elongated “stretched” in TM treated HUVECs, indicating activation of the endothelium, while the control cells appeared to form a normal “cobblestone” (**Figure.4, A**). qPCR data demonstrated a significant increase in the ER stress gene expression profiles for all the genes tested on tunicamycin-treated HUVECs compared to untreated, DMSO-treated controls and CFZ-treated HUVECs (**Figure.4, B**). This finding was further confirmed by western blot analysis (**Figure.5, A&B**). This data shows tunicamycin induced ER-stress on HUVECs, but carfilzomib did not achieve this at the investigated experimental conditions.


A
0HR
48HR / DMSO
24HR / TM
48HR / TM

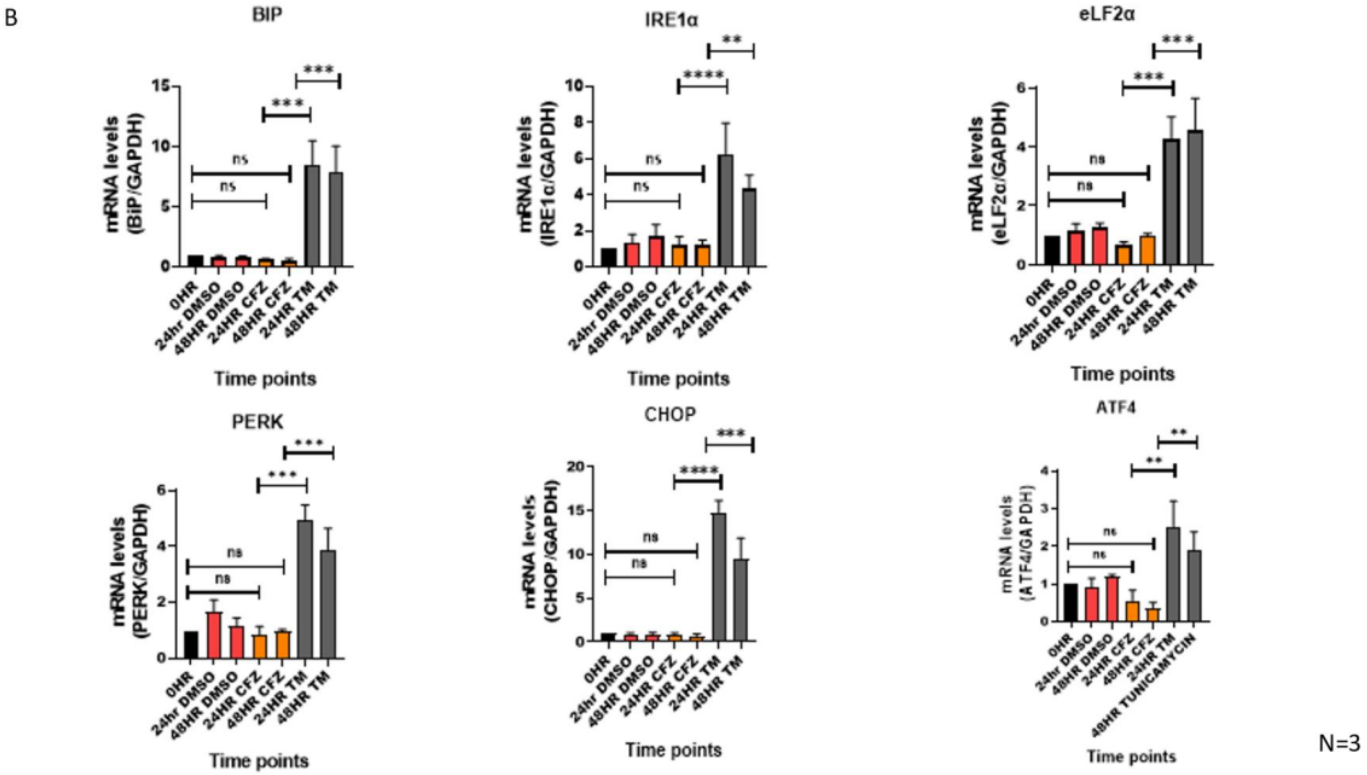
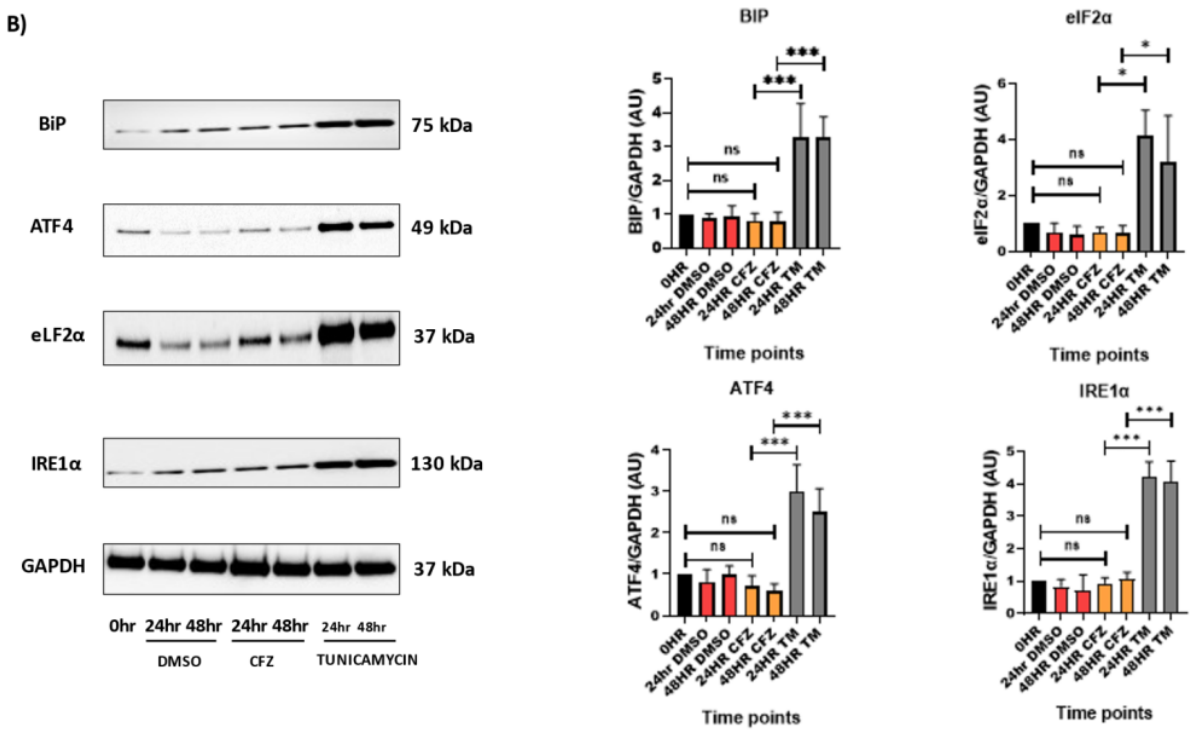


Figure 4: Tunicamycin treatment induces ER stress in HUVECs. HUVECs were treated with 750nM of CFZ, DMSO for 1hour, and 10μg/ml tunicamycin for 24hr, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. **A)** Morphological observation

using light microscope (10X lens) Cells were harvested to extract RNA at 0hr, 24hr, and 48hr. cDNA was prepared from RNA to quantify mRNA expression. **B)** q-PCR The expression level of principal's ER stress mRNA markers (BIP, PERK, eLF2α, ATF4, CHOP, and IRE1α) in tunicamycin treated HUVECs and controls. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of mRNA and protein species between CFZ and DMSO cells are compared using one-way ANOVA. Significant: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.


B)
BiP
ATF4
eLF2α
IRE1α
GAPDH
75 kDa
49 kDa
37 kDa
130 kDa
37 kDa
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DMSO
CFZ
TUNICAMYCIN
BIP
BIP/GAPDH (AU)
eIF2α
eIF2α/GAPDH (AU)
ATF4
ATF4/GAPDH (AU)
IRE1α
IRE1α/GAPDH (AU)
ns
***
*
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
24HR TM
48HR TM
Time points

N=3

Figure 5: ER stress protein expression of tunicamycin treated HUVECs compared to CFZ treated HUVECs and controls HUVECs were treated with 750nM of CFZ and DMSO for 1hour, 10 μg/ml tunicamycin for 24hr, followed by washing the cells with PBS and resuspended them in fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. Western blot was used to determine the protein level. A) Western Blots bands of BIP (75 kDa), ATF4 (49 kDa), eLF2α-p (37 kDa), IRE1α-p (130 kDa), and GAPDH (37 kDa). B) Density quantification of western blots. DMSO;

Dimethyl sulfoxide, CFZ; carfilzomib; TM; tunicamycin. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of mRNA and protein species between tunicamycin and CFZ cells were compared using one-way ANOVA. Significant: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

3.2.4 carfilzomib does not induce an accumulative effect on the endothelium

In clinical practice, carfilzomib is administered over a 28-day treatment cycle over two consecutive days each week and infused intravenously in MM patients over a 30-minute infusion rate. Since our previous CFZ experimental setting could not induce ER stress in the endothelium; therefore, we hypothesised that two consecutive days of HUVECs stimulation with carfilzomib could build up carfilzomib cytotoxic effect on the endothelium; thus induce ER stress in HUVECs and overcome the limitation of single dosage experiment. The cells were stimulated twice with 750nM of CFZ in two consecutive days for one hour/ 24 hr recovery with fresh EBM-2 medium after each CFZ treatment. The data obtained showed an evident accumulation of the proteasome inhibition effect in the endothelium once the cells were treated twice with CFZ for two following days (**Figure. 6A**). This phenomenon was not seen in the earlier single dosage of CFZ, where the ubiquitination expression returned to normal after 48 hr recovery. This finding indicates that stimulating HUVECs with CFZ in similar conditions to the clinical setting enhances the proteasome inhibition ability of CFZ in the endothelium. However, this accumulation of proteasome inhibition in the endothelium was not adequate to trigger the induction of ER stress in the endothelium. The expression of both ER stress markers (BiP and P58) was comparable to both non treated and vector control cells either after single pules or double pules with CFZ (**Figure. 6B**). Hence, all these data indicate that even though two consecutive days of stimulation with CFZ has increased the ubiquitin expression in the endothelial cells, it did not induce ER stress activation in HUVECs; implying that the potential ability of HUVECs to escape the pathological impact of CFZ under the current experimental conditions.

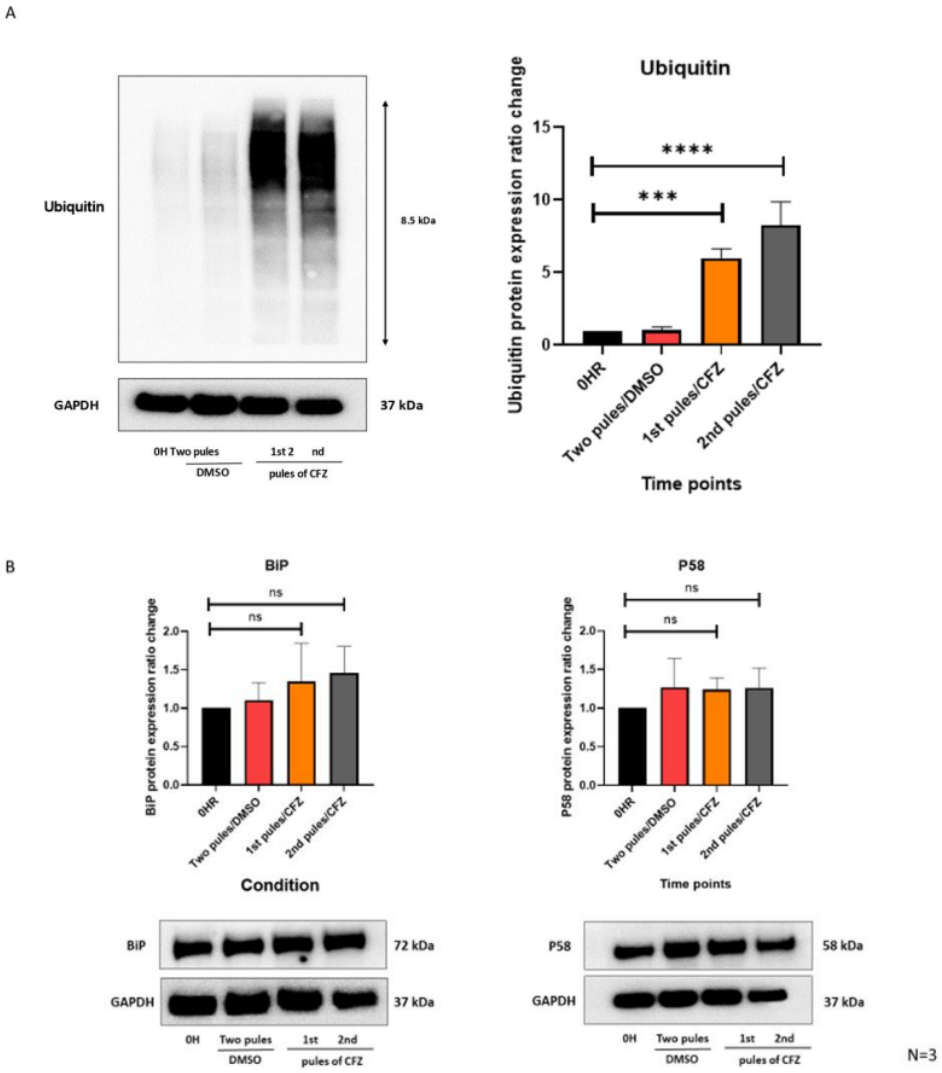


Figure 6: Carfilzomib does not induce an accumulative effect on the endothelium. HUVECs were treated twice with 750nM of CFZ and DMSO for 1hour, for two consecutive days, followed by washing the cells with PBS, resuspended them in fresh EBM-2 medium and left them to recover for 24 hr after each CFZ treatment Cells were harvested to extract protein at 0hr, 24hr after 1st and 2nd DMSO or CFZ treatment. Western blot was used to determine the protein level. ***A***) Western Blots analysis of ubiquitin-

protein level expression. **B)** Density quantification of western blot. **C)** Western Blots bands of BIP (75 kDa), P58 (58 kDa), and GAPDH (37 kDa) and Density quantification of western blots. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of mRNA and protein species between 0hr, DMSO and CFZ cells were compared using one-way ANOVA. Significant: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

2.5 Increased different concentrations of CFZ did not induce ER stress in the endothelium

Since there was no induction of ER stress in the endothelium either with a single/ accumulative dosage of 750nM CFZ, we hypothesised that using a higher dosage of CFZ could induce ER stress in the endothelium. For this purpose, we treated the cells with three different increased concentrations of CFZ; 750nM, 1500nM, and 3000nM for 1 hr and followed by 24hr recovery. All these treatment dosages were used similar to the clinical dosage used in MM patients. Interestingly, there was no induction of ER stress in the endothelium among all the concentrations applied on the cells (**Figure. 7**). Comparable BiP revealed this, and P58 in the CFZ treated cells compared to non-treated, and DMSO controls. This finding indicates that CFZ cannot trigger the activation of ER stress in the endothelium, even with the usage of a higher concentration of the drug.

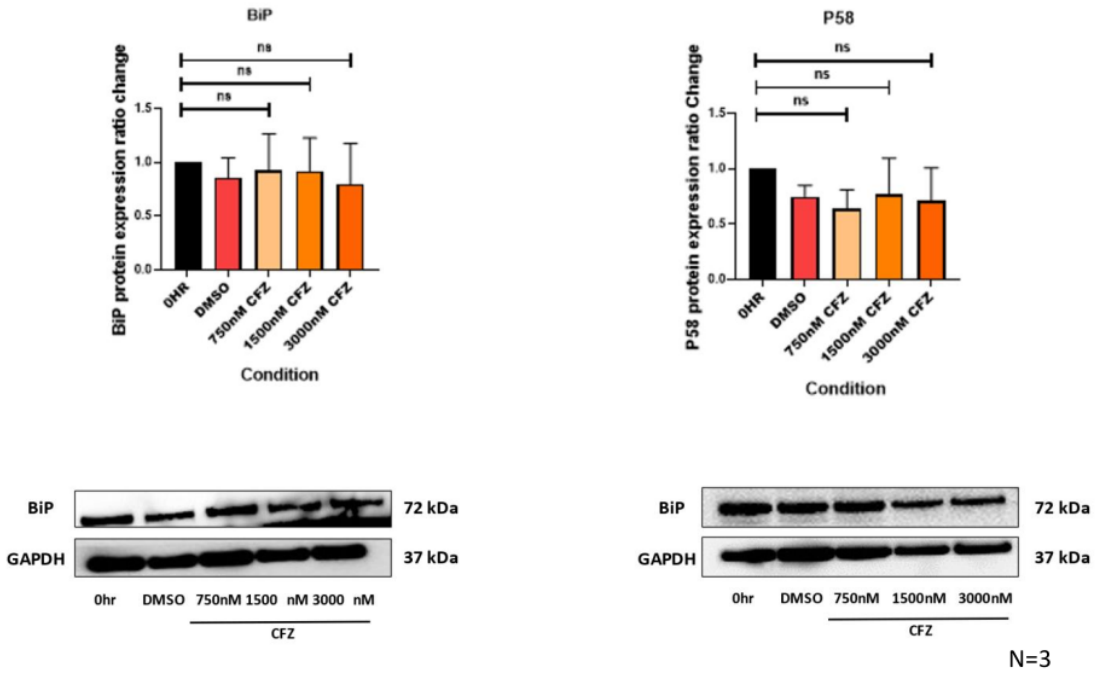


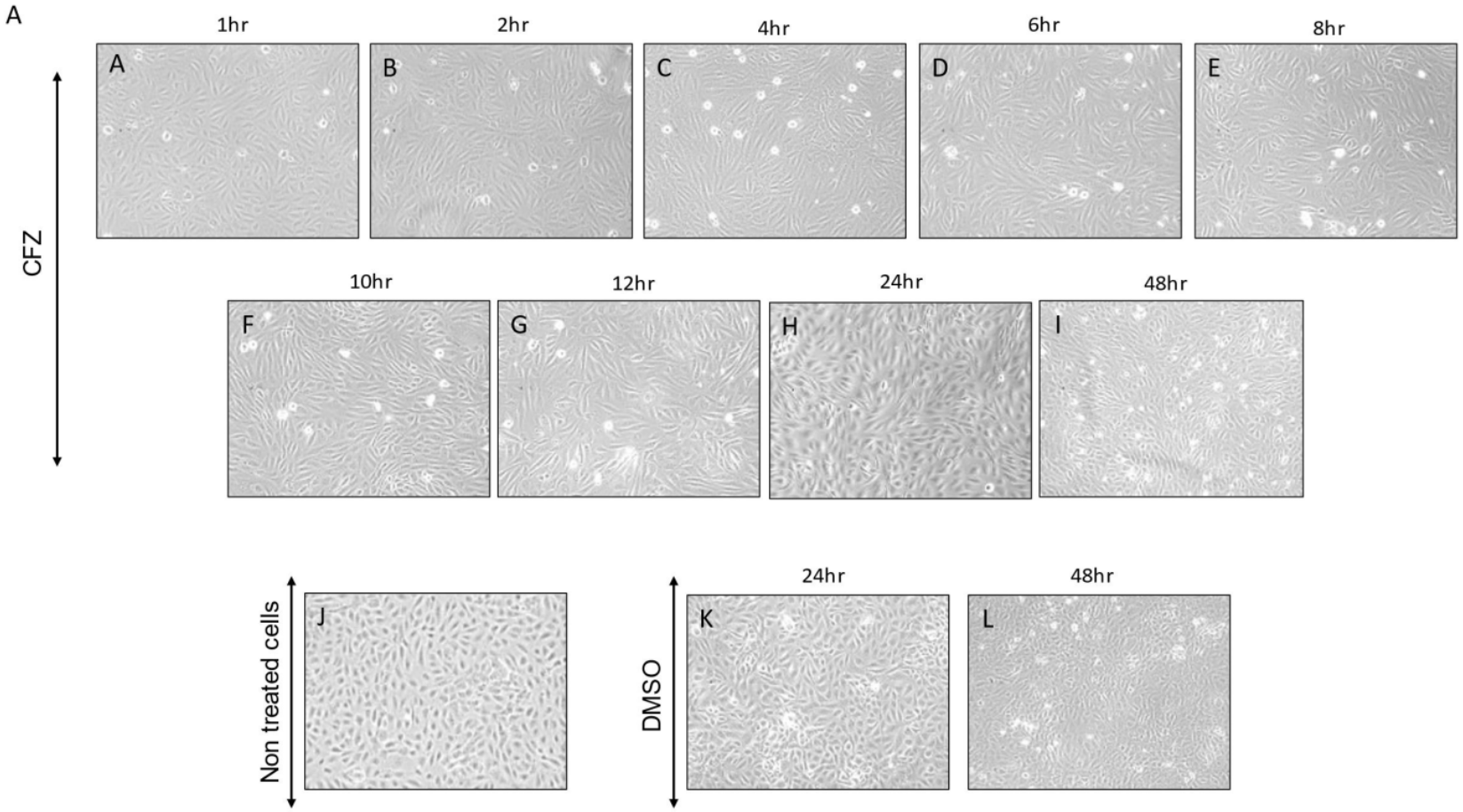
Figure 7: higher concentration of CFZ is not inducing ER stress in the endothelium. HUVECs were treated with 750nM, 1500nM, and 3000nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr,

24hr and western blot was used to determine the extent of ER stress. Western Blots analysis of BiP and P58 proteins level expression and Density quantification of western blot. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of mRNA and protein species between 0hr, DMSO and CFZ cells were compared using one-way ANOVA. Significant: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

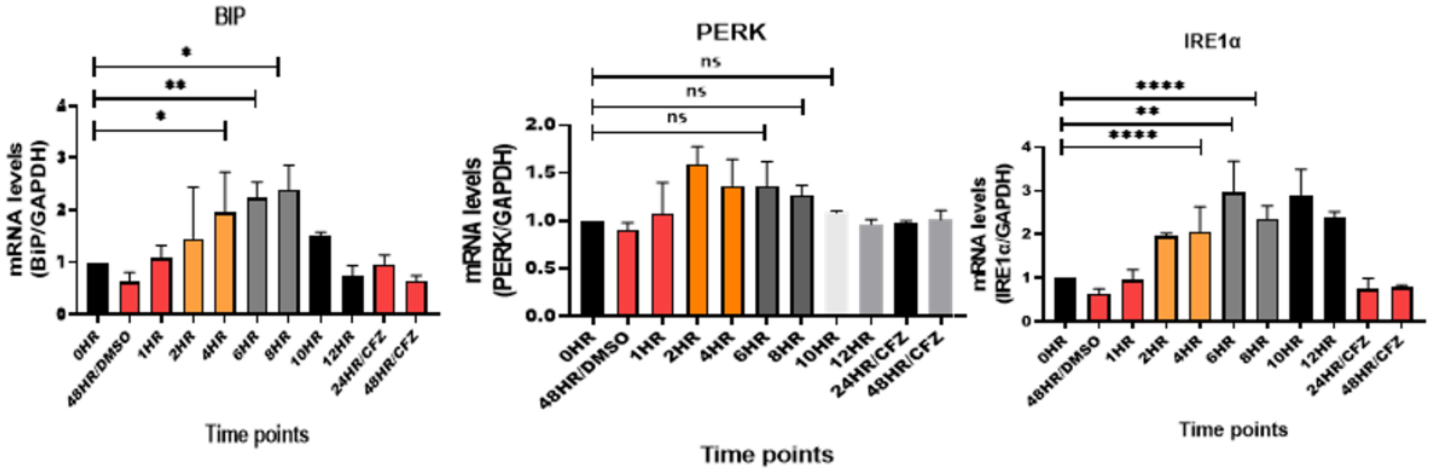
3..2.6 Carfilzomib is inducing ER stress in HUVECs at earlier recovery time points.

Since none of the earlier experiments has shown an ER stress induced by CFZ, and since there was an apparent morphological alteration of HUVECs at 24 hr recovery; therefore, we hypothesised that CFZ could induce a potential ER stress in HUVECs at earlier recovery time points which have not been detected at the earlier experimental time setting. For this purpose, the cells were treated with 750 nM of CFZ for 1 hr, washed with PBS, and resuspended in fresh EBM-2 medium for selected nine recovery time points. These time points were selected as 1hr, 2hr, 4hr, 6hr, 8hr, 10 hr, 12 hr, 24hr, and 48 hr, respectively. The light microscope observation showed that there was a progressive morphological change in CFZ treated cells. The morphological alteration of HUVECs started after 1 hr recovery and lasted up to 24 hr recovery. These morphological abnormalities were noticed as the cells became elongated and stretched, which was more apparent than the 10hr and 12 hr HUVECs recovery time points (**Figure.8A, E&F**). However, this morphological change became less potent at 24hr recovery and almost returned to normal at 48 hours (**Figure.8A, H&I**). In general, the control cells appeared to form a typical "cobblestone" pattern while the CFZ treated cells appeared more elongated and "stretched" throughout the essay, indicating a potential activation of the endothelium that progressed through the assay. In equivalence, an increase of some of the ER stress markers genes was observed within 24 hr recovery in the CFZ treated

HUVECs. There was a significant induction of both BiP and IRE1α genes between 2hr-10 hr post-treatment recovery. There was mild non-significant upregulation of PERK and ATF-4 genes between 2hr-8hr and 2hr-4hr post-treatment recovery, respectively. These data indicate that an earlier induction of ER stress happened in the endothelium at earlier time points recovery, followed by cellular recovery started at 24 hr recovery and onward (**Figure.8B**).


A
1hr
2hr
4hr
6hr
8hr
A
B
C
D
E
CFZ
10hr
12hr
24hr
48hr
F
G
H
I
Non treated cells
J
DMSO
24hr
48hr
K
L

B

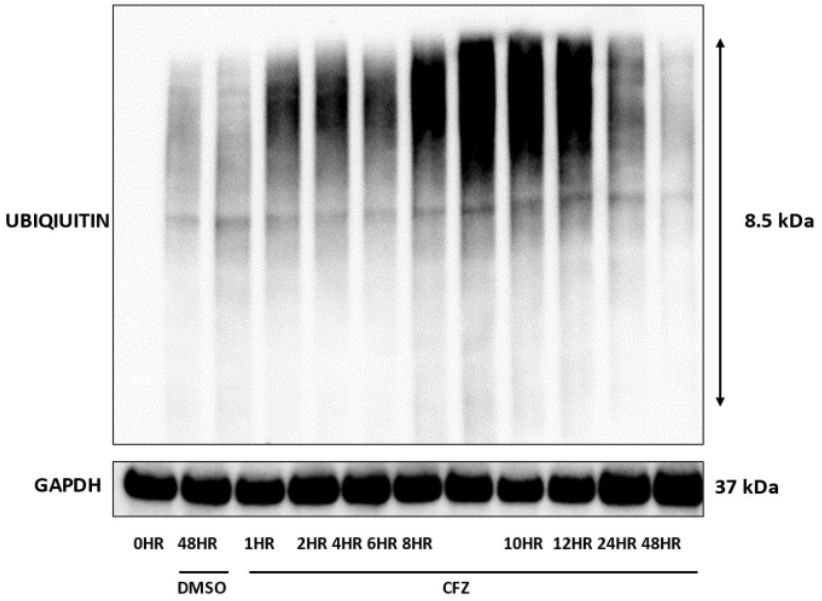

BIP
mRNA levels (BiP/GAPDH)
*
**
*
0HR
48HR/DMSO
1HR
2HR
4HR
6HR
8HR
10HR
12HR
24HR/CFZ
48HR/CFZ
Time points
PERK
mRNA levels (PERK/GAPDH)
ns
ns
ns
Time points
IRE1α
mRNA levels (IRE1α/GAPDH)
****
**
****
Time points

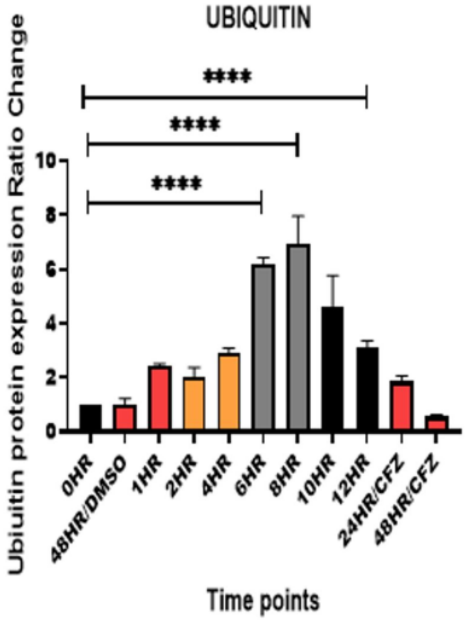
N=3

Figure 8: CFZ is inducing ER stress induction at early recovery time points. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. qPCR was used to determine the extent of ER stress. ***A)*** Morphological observation using a light microscope (10X lens). Western Blots analysis of ubiquitin-protein level expression. **B)** The expression level of mRNA of principal's ER stress markers (BIP, PERK, eLF2α, ATF4, CHOP, and IRE1α) in CFZ treated HUVECs and controls**.** DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

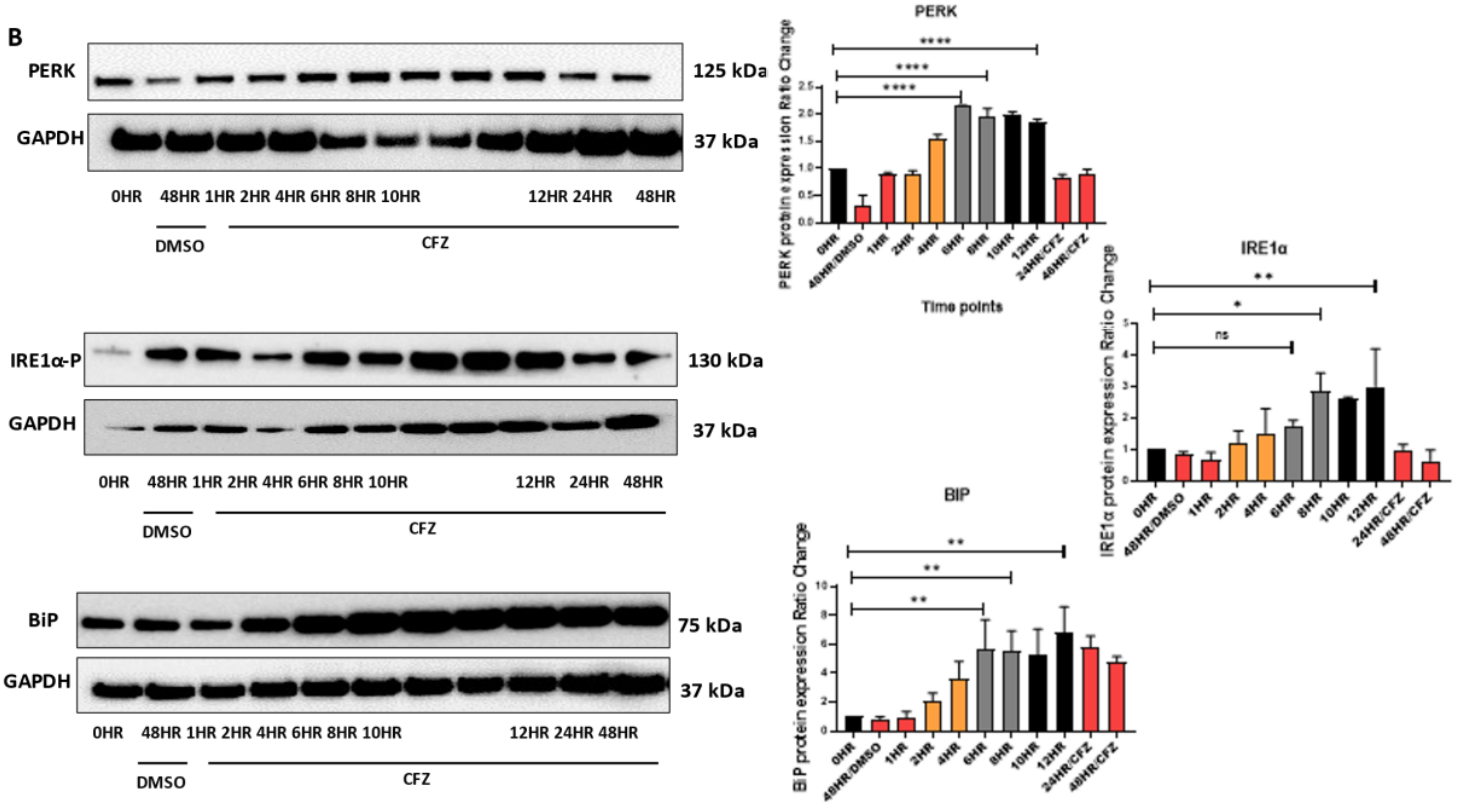
The protein analysis was correlated with our genomic findings apart from BiP protein expression. There was gradual upregulation of the ubiquitin expression in the CFZ treated HUVECs started from 1hr recovery and reached the peak at 8hr recovery, followed by gradual reduction of the ubiquitination expression until the cells reached complete remission at 48 hr recovery (**Figure.9A**). There was a gradual induction of both PERK and IRE1α started from 4hr recovery and reached the peak at 12hr recovery, followed by gradual reduction of these proteins expression until reaching complete remission at 24 hr recovery (**Figure.9B**). These data indicate that an earlier induction of ER stress happened in the endothelium at earlier time points recovery, followed by cellular recovery started at 24 hr recovery and onward. However, the western blot analysis of BiP protein expression showed that there was a significant upregulation of this protein in the CFZ treated HUVECs started from 2hr recovery and constantly last, indicating a permanent lasting of the ER stress activation in the endothelium. The latest finding in this experiment was contrasting what has been observed earlier (**Figure.3**), where there was no upregulation of BiP at either 24hr or 48hr recovery, which was required further investigation to reveal the conflicting outcomes.

A


UBIQIUITIN
8.5 kDa
GAPDH
37 kDa
0HR 48HR 1HR 2HR 4HR 6HR 8HR 10HR 12HR 24HR 48HR
DMSO
CFZ


UBIQUITIN
Ubiuitin protein expression Ratio Change
****
****
****
10
8
6
4
2
0
0HR
48HR/DMSO
1HR
2HR
4HR
6HR
8HR
10HR
12HR
24HR/CFZ
48HR/CFZ
Time points

N=3

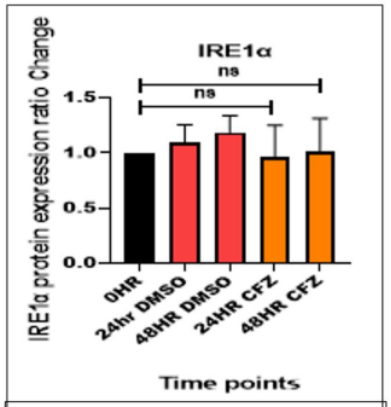

B
PERK
GAPDH
125 kDa
37 kDa
0HR 48HR 1HR 2HR 4HR 6HR 8HR 10HR 12HR 24HR 48HR
DMSO
CFZ
IRE1α-P
GAPDH
130 kDa
37 kDa
0HR 48HR 1HR 2HR 4HR 6HR 8HR 10HR 12HR 24HR 48HR
DMSO
CFZ
BiP
GAPDH
75 kDa
37 kDa
0HR 48HR 1HR 2HR 4HR 6HR 8HR 10HR 12HR 24HR 48HR
DMSO
CFZ
PERK
PERK protein expression Ratio Change
Time points
IRE1α
IRE1α protein expression Ratio Change
BIP
BiP protein expression Ratio Change

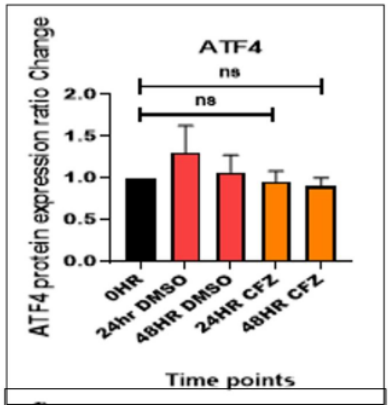
N=3

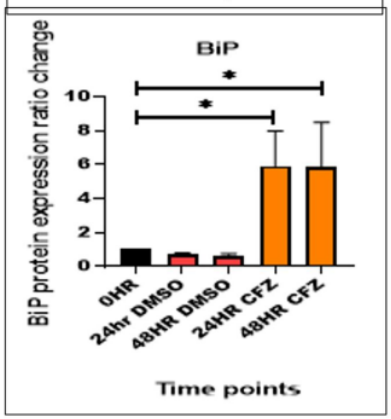
Figure 9: CFZ is inducing ER stress induction at early recovery time points. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. qPCR was used to determine the extent of ER stress. ***A)*** Western Blots analysis of ubiquitin-protein level expression and Density quantification of western blot. **B)** Western Blots analysis of some ER stress markers (PERK, BiP, IRE1α) level expression and Density quantification of western blot. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *P < 0.05, **P < 0.01, ***P < 0.001

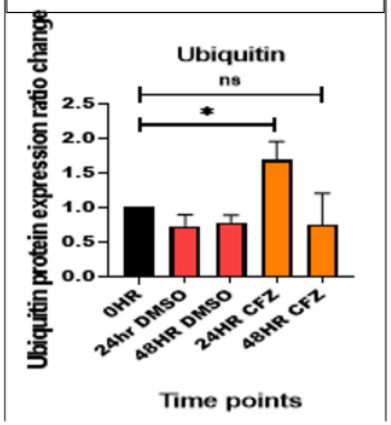
3.2.7 CFZ is inducing BiP upregulation but no other ER stress markers

BiP/GRP78, an essential ER chaperone to detect misfolded proteins(12). Under basal conditions, BiP binds to the three ER stress sensors' (IRE1α, ATF6, and PERK) luminal domains, thus inhibiting their activation (13). However, BiP dissociates from the sensors and strongly binds to the exposed hydrophobic domains of the proteins when they accumulate in the ER (14). The phosphorylation and activation of these sensors initiate pathways signalling to establish an adaptive cellular response that resolves ER stress and restores cellular protein homeostasis. Since there was a conflicting outcome regarding BiP protein expression in my previous findings, indicating a potential activation of ER stress by CFZ in the endothelium, or it could be due to experimental error. Therefore, I repeated the initial two-time points (24hr and 48hr) recovery experiment to identify any potential diversity from my earlier 24hr and 48hr recovery time points. Interestingly, I found a significant upregulation in BiP expression in the recent experiment (**Figure.10**), which was not detected in my previous results (**Figure.3**). This conflicting data could be experimental error, sub hidden ER activation, which was not detected in my previous experiment, or a BiP protein particular mechanism.


IRE1α
ns
ns
IRE1α protein expression ratio Change
1.5
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points


ATF4
ns
ns
ATF4 protein expression ratio Change
2.0
1.5
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points


BiP
*
*
BiP protein expression ratio change
10
8
6
4
2
0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points


Ubiquitin
ns
*
Ubiquitin protein expression ratio change
2.5
2.0
1.5
1.0
0.5
0.0
0HR
24hr DMSO
48HR DMSO
24HR CFZ
48HR CFZ
Time points

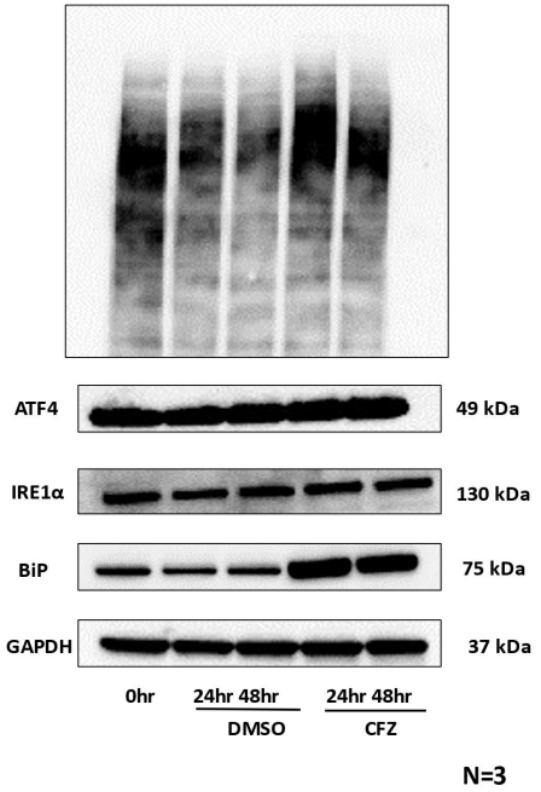
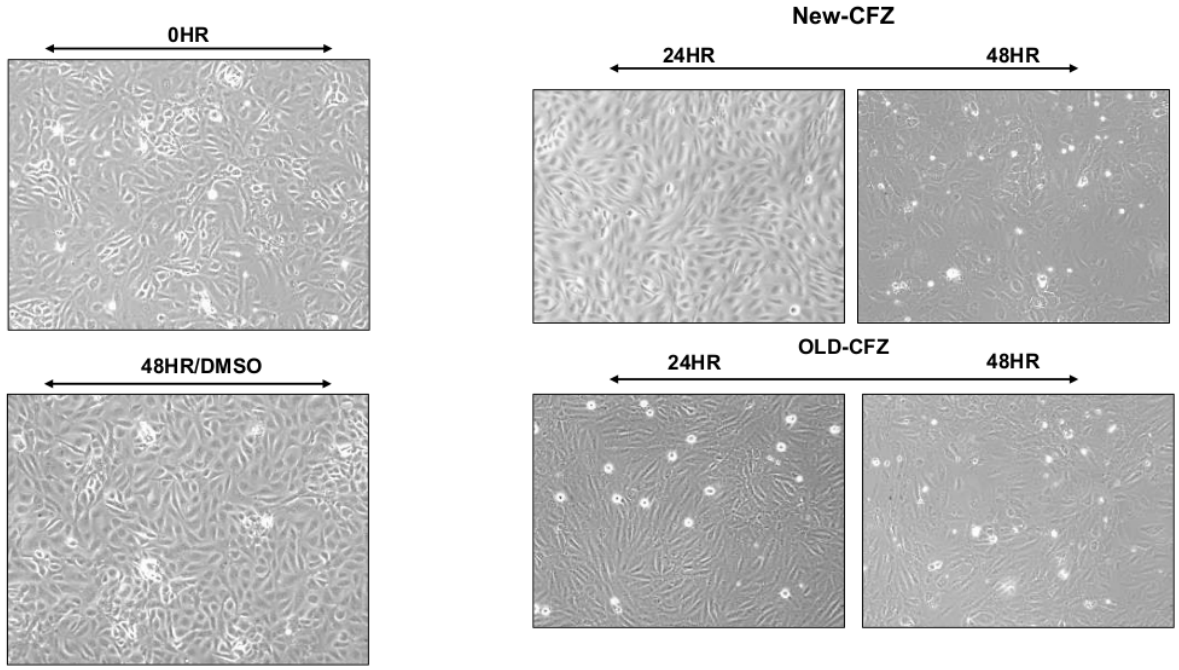

ATF4
49 kDa
IRE1α
130 kDa
BiP
75 kDa
GAPDH
37 kDa
0hr
24hr 48hr
DMSO
24hr 48hr
CFZ
N=3

Figure 10: CFZ is inducing BiP upregulation but no other ER stress markers. HUVECs were treated with 750nM of CFZ for 1 hour, followed by washing the cells with PBS and resuspending them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. Western Blots analysis of some of the ER stress markers (BiP, IRE1α, and ATF-4) protein level expression and Density quantification of western blot. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *P < 0.05, **P < 0.01, ***P < 0.001

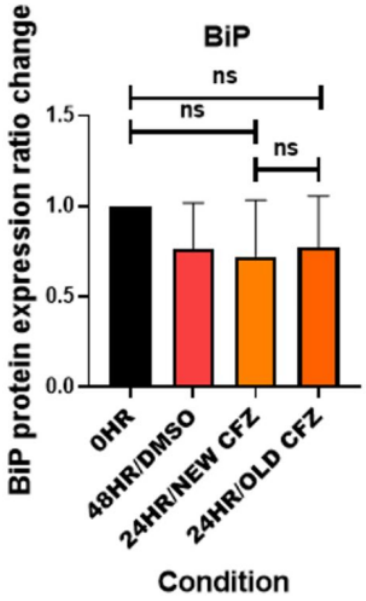
3..2.8 Resolving the conflicting in the BiP protein expression data

The data obtained previously showed that there was a conflict about BiP protein expression. My earlier findings showed no induction of BiP protein expression at either 24hr or 48hr recovery; however, the later experiments showed significant induction of BiP at these time points. Therefore, we established a detailed backward root data analysis to identify the potential experimental difference that could have happened earlier caused these conflicting outcomes. The first thing we noticed was a difference in the drug patch used in the diverse experiments. The new patch of the drug purchased and used after the initial experiments showed no upregulation of BiP, indicating potential involvement of the drug source diversity as a potential cause of the raised incident. Therefore, we conducted an experiment where HUVECs were treated in parallel for 1hr using the new and old patches of the drug. The cells then were resuspended in a fresh EBM-2 medium and left to recover for 24 hr. Surprisingly, the western blot analysis showed that there was no induction of BiP expression in both “the old” and “the new” patches of the drug (**Figure.11**), indicating that it was not because of CFZ ability to induce ER stress but it was most likely because of experimental diversion among the experiments.

A



B


BiP
ns
ns
ns
1.5
1.0
0.5
0.0
BiP protein expression ratio change
0HR
48HR/DMSO
24HR/NEW CFZ
24HR/OLD CFZ
Condition

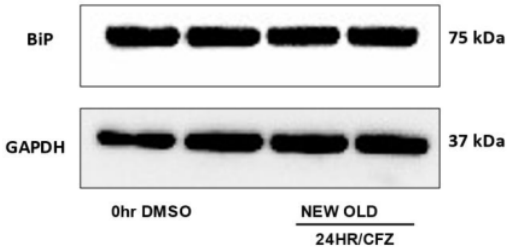

BiP
75 kDa
GAPDH
37 kDa
0hr DMSO
NEW OLD
24HR/CFZ

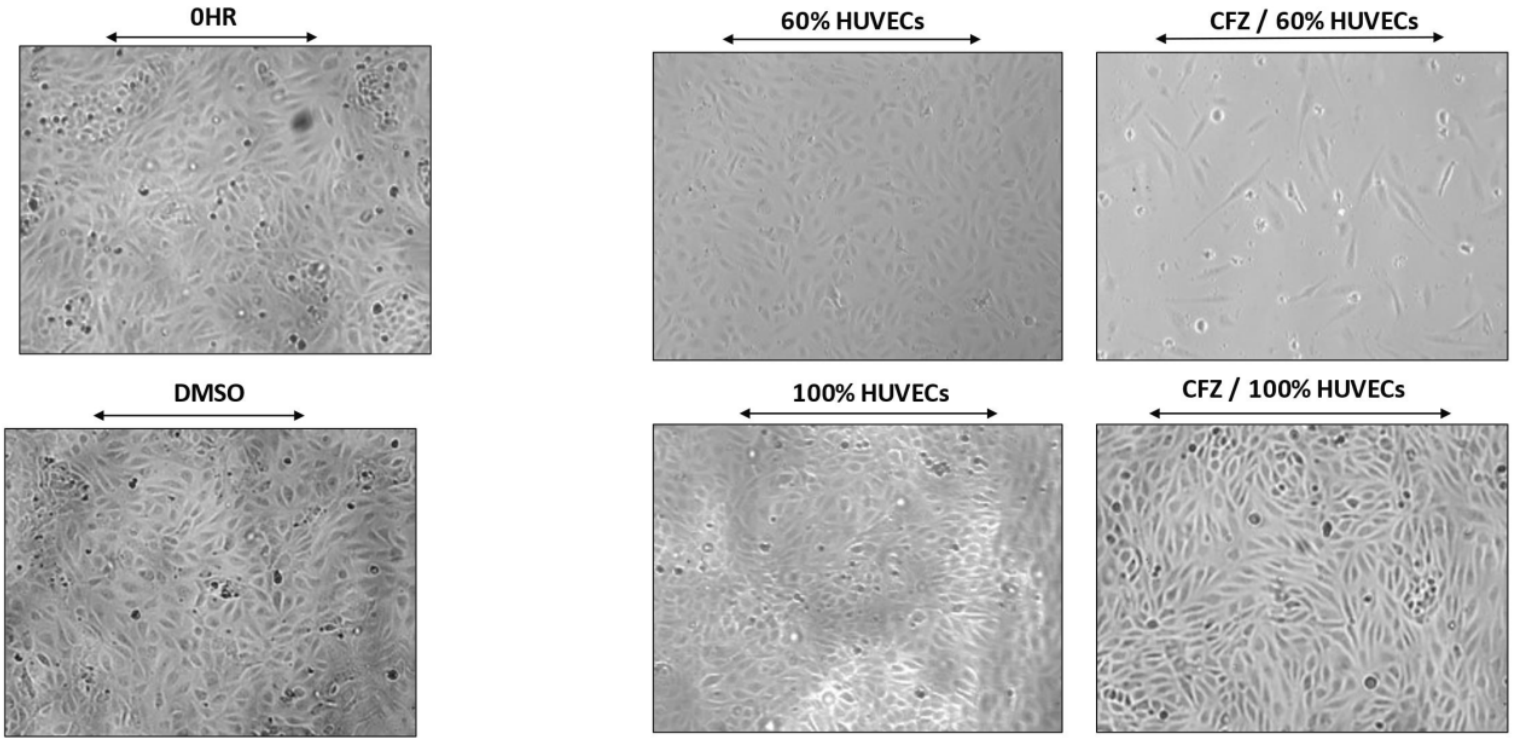
Figure 11: ER stress was not induced by Both old and new CFZ patches. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr, 24hr, and 48hr. Western blot was used to determine the extent of ubiquitination. ***A)*** Morphological observation using a light microscope (10X lens). **B)** Western Blots analysis of BiP-protein level expression and density quantification of western blot. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of BiP between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Since both drugs showed a similar outcome regarding the absence of BiP induction at 24 hr recovery, the cells used in all the experiments were cultured from the same patch of the cells. Therefore, we carful revised all the potential experimental diversity that could occur during the experiments' process, which had led us to identify the potential source of the conflicting data. When we checked all the morphological observations among all the experiments, we found that there was a diversion in the cell confluency when the experiments were initiated. We found that the confluency of the cells was less than 100 % (60-80%) in all the experiments where BiP was up-regulated after 24 hr recovery. Indeed, this was conflicting with our initial established experimental protocol that indicates the 100% confluency of the cells as a mandatory requirement to initiate the experiment.

On the other hand, we found that the cells were 100 % confluent in all the experiments when were BiP was not up-regulated. Therefore, we hypothesised that non fully confluent cells are rapidly growing cells and more protein demanding cells; thus, they are more deliciated and susceptible to proteasome inhibitors. For this purpose, we established a different confluence CFZ experiment. The cells were cultured in two different cellular confluencies (60% and 100%), followed the cells were treated with 750 nM of CFZ for 1he, resuspended in fresh EBM-2 medium and then left to recover for 24 hr. As expected, the 60 % confluent cells showed a remarkable morphological change and elongation, which was mild in 100 % confluent cells (**Figure.12A**). In addition, there was more intensity of ubiquitination expression in the fewer confluence cells than 100 % confluence cells, indicating more ubiquitination of the cytosol floating proteins: thus, more demanding of the protein-producing in the 60% confluence cells. (**Figure.12B**). Notably, there was a significant upregulation of BiP expression in 60% confluence HUVECs only, indicating that the ER stress caused by CFZ is potential growing HUVECs. However, in the typical human vascular system, the endothelium forming a monolayer structure (100 % confluence), this making the ER stress-induced in 60% confluence HUVECs is less suitable in physiological conditions. Taken all

together, according to our findings, we can conclude that the usage of 750nM of CFZ for 1hr is not adequate to induce ER stress in the full confluence HUVECs (monolayer structure).

A



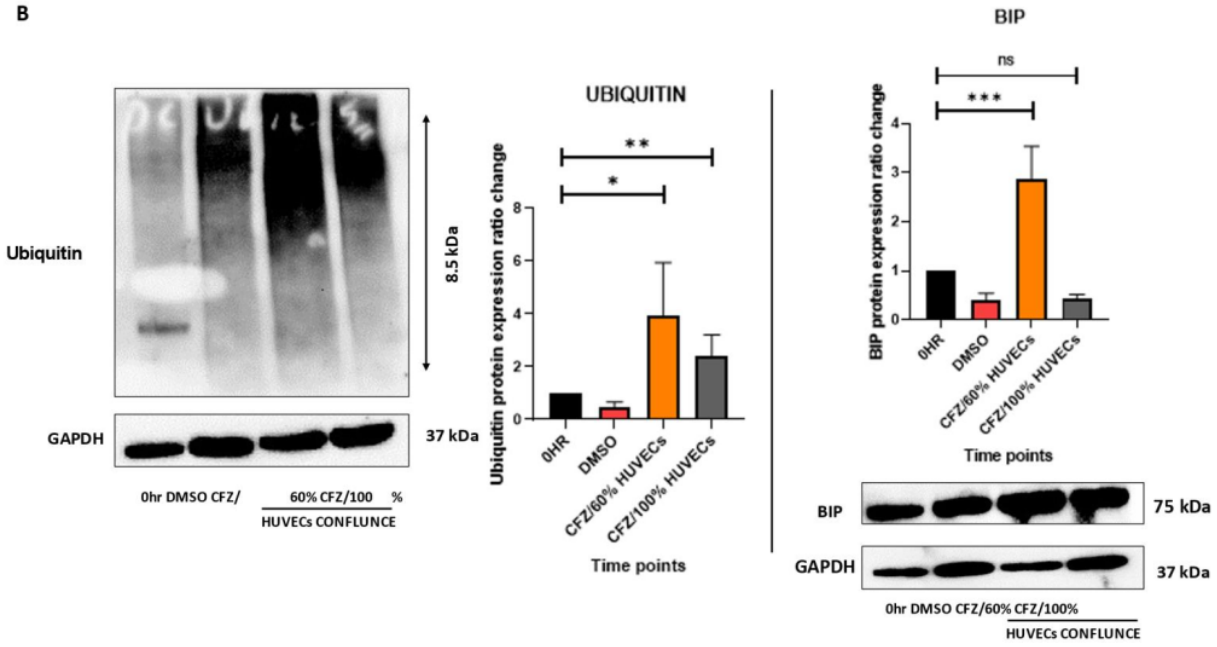
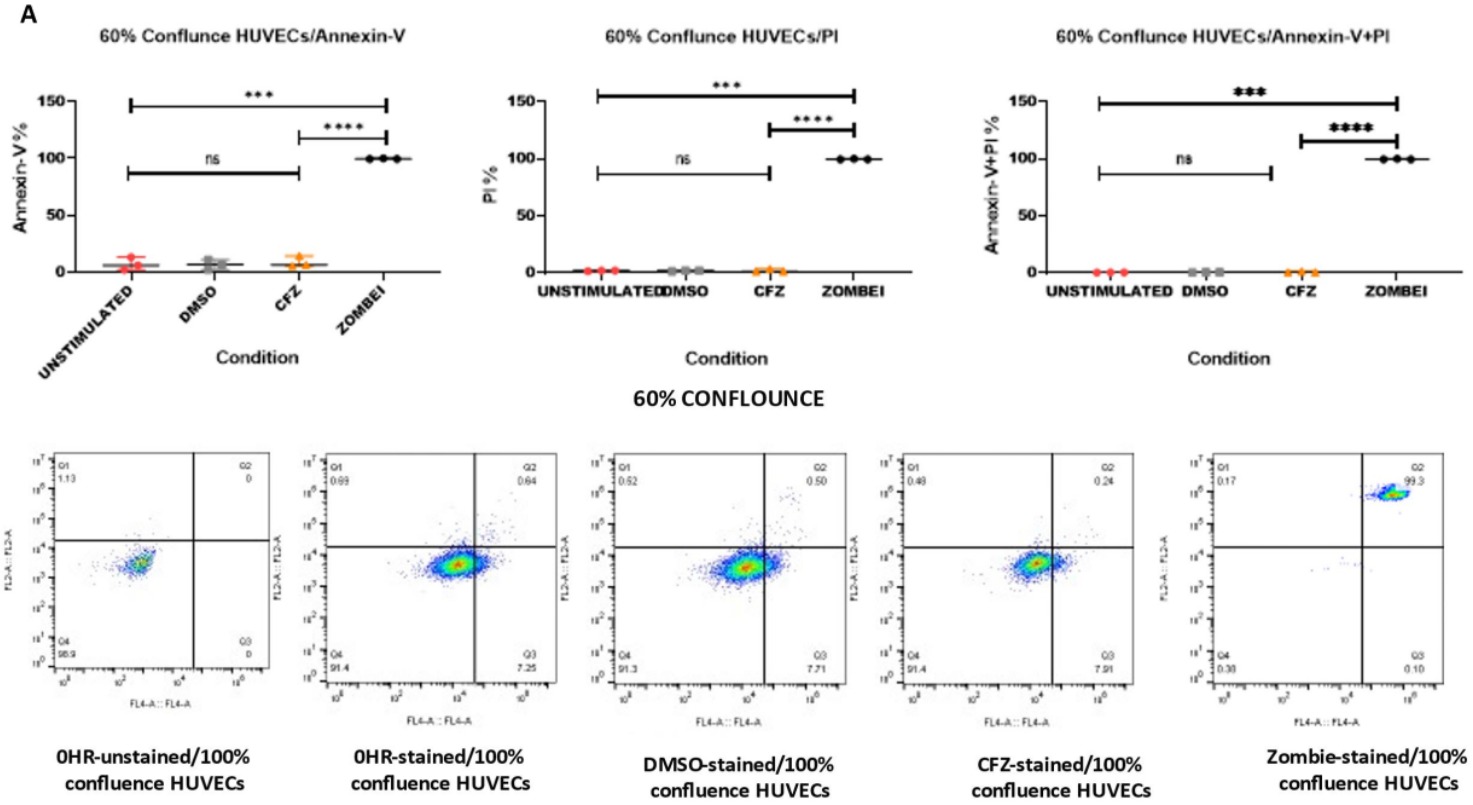

B
Ubiquitin
8.5 kDa
GAPDH
37 kDa
0hr DMSO CFZ/
60% CFZ/100 %
HUVECs CONFLUNCE
UBIQUITIN
Ubiquitin protein expression ratio change
**
*
0HR
DMSO
CFZ/60% HUVECs
CFZ/100% HUVECs
Time points
BIP
ns
***
BIP protein expression ratio change
0HR
DMSO
CFZ/60% HUVECs
CFZ/100% HUVECs
Time points
BIP
75 kDa
GAPDH
37 kDa
0hr DMSO CFZ/60% CFZ/100%
HUVECs CONFLUNCE

Figure 12: CFZ induces BiP upregulation in 60 % confluence HUVECs but not in 100 % confluence HUVECs. HUVECs were cultured in two different confluences, 60% and 100%, followed the cells were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in fresh EBM-2 medium. Cells were harvested to extract protein at 0hr and 24hr. ***A***) Morphological observation using a light microscope (10X lens). ***B***) Western Blots analysis of ubiquitin and BiP proteins level expression and Density quantification of western blots. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *P < 0.05, **P < 0.01, ***P < 0.001

3.2.9 CFZ did not induce apoptosis either in 60% or 100% confluence HUVECs

Even though our findings showed the lack of CFZ ability to induce ER stress in the fully confluence HUVECs, there was considerable morphological alteration progress along with the experiments. Therefore, we asked if other mechanisms could cause endothelium damage by CFZ, such as cellular apoptosis. For this purpose, we conducted another different confluence experiment (60% and 100% confluence HUVECs), and we examined the expression of the well-known apoptotic markers (Annexin-V and PI) using both flow cytometry and immunostaining. Interestingly, the flow cytometry study revealed that the percentage of each apoptotic marker or combined was similar in CFZ treated HUVECs compared to the untreated and DMSO controls among all the different confluence cells (Figure **13A&B**). This finding is indicating that the absence of apoptosis triggering by CFZ in the endothelium. The flow cytometry finding was conformed using Annexin-V immune staining. Inconsistent with the flow cytometry finding, the morphological observation indicated a comparable fluorescence intensity among all different confluence CFZ treated cells and their controls (**Figure.14**). Considering all these findings, the endothelium apoptosis caused by CFZ was not achievable in our experiment, though with the usage of less confluent cells, indicating it was not an applicable cause to describe the potential effect of CFZ on the endothelium.



0HR-unstained/100% confluence HUVECs

0HR-stained/100% confluence HUVECs

DMSO-stained/100% confluence HUVECs

CFZ-stained/100% confluence HUVECs

Zombie-stained/100% confluence HUVECs

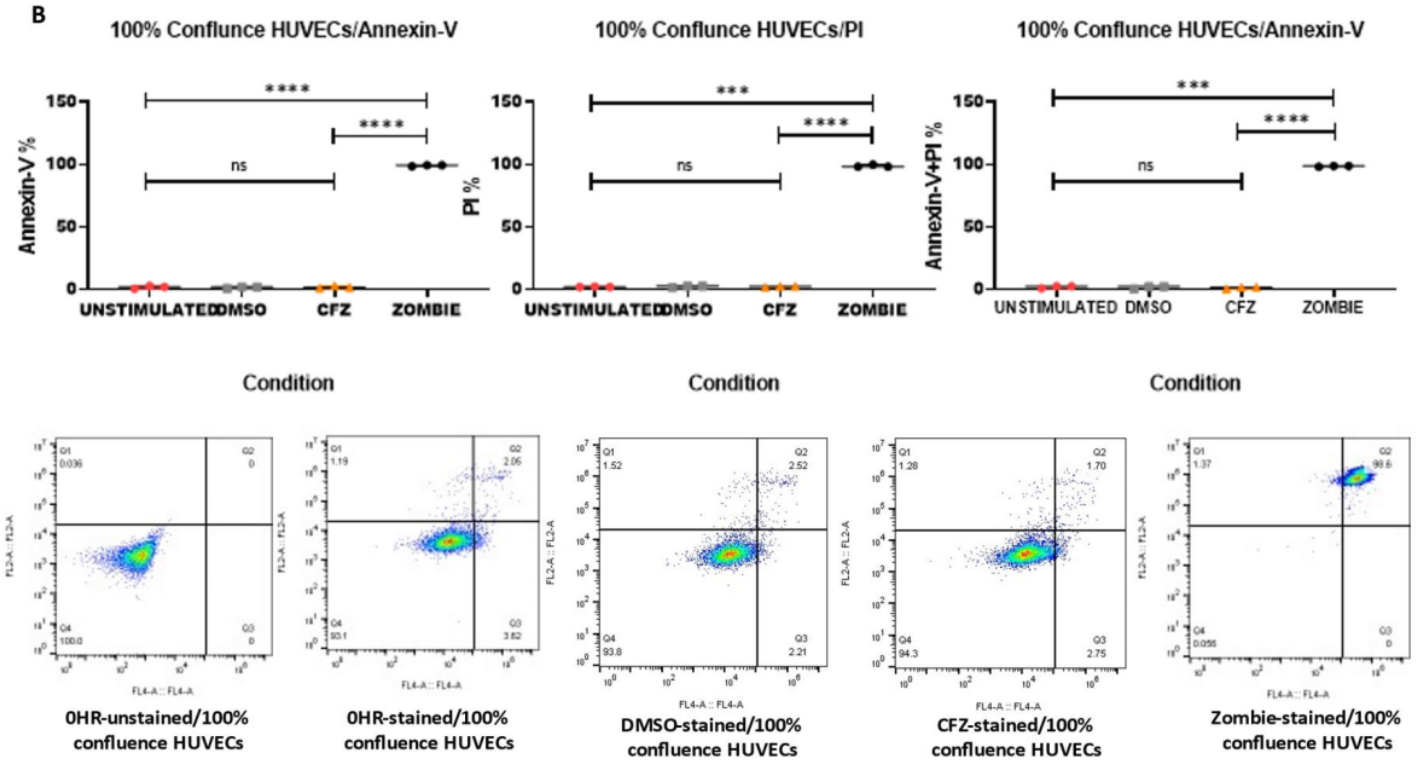

B
100% Conflunce HUVECs/Annexin-V
100% Conflunce HUVECs/PI
100% Conflunce HUVECs/Annexin-V
Annexin-V %
PI %
Annexin-V+PI %
****
***
ns
UNSTIMULATED
DMSO
CFZ
ZOMBIE
Condition
0HR-unstained/100% confluence HUVECs
0HR-stained/100% confluence HUVECs
DMSO-stained/100% confluence HUVECs
CFZ-stained/100% confluence HUVECs
Zombie-stained/100% confluence HUVECs

Figure 13: CFZ did not trigger apoptosis in 60% or 100% confluence HUVECs. HUVECs were cultured in two different confluences, 60% and 100%, followed the cells were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in fresh EBM-2 medium. Cells were harvested to extract protein at 0hr and 24hr. ***A)*** Annexin-V and PI flow cytometry analysis for 60% confluence cells. **B)** Annexin-V and PI flow cytometry analysis for 100% confluence cells. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM.

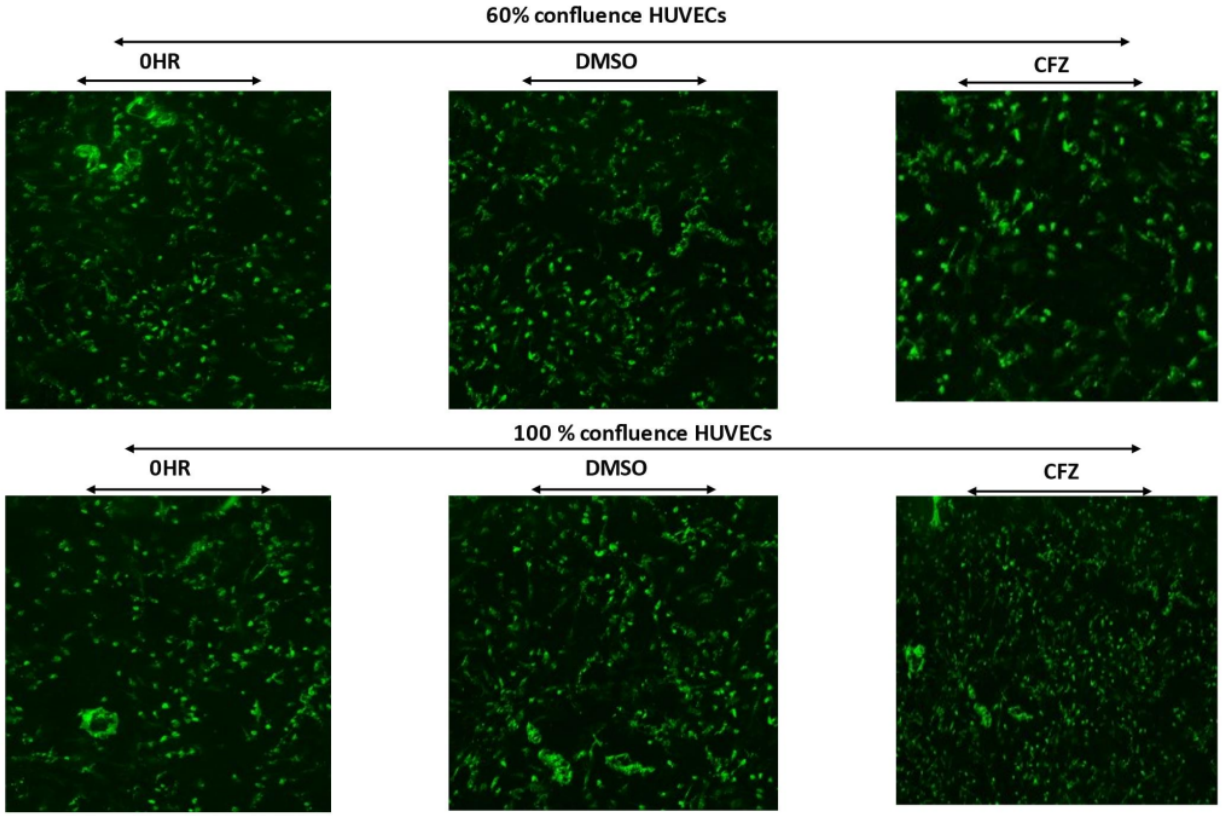
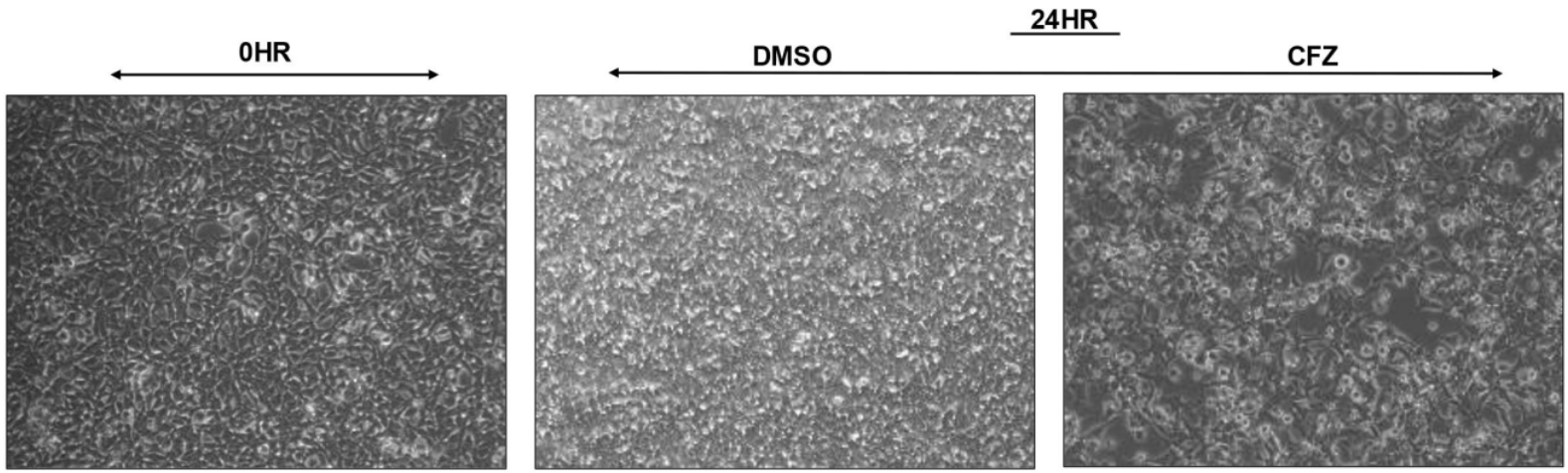

60% confluence HUVECs
0HR
DMSO
CFZ
100 % confluence HUVECs
0HR
DMSO
CFZ

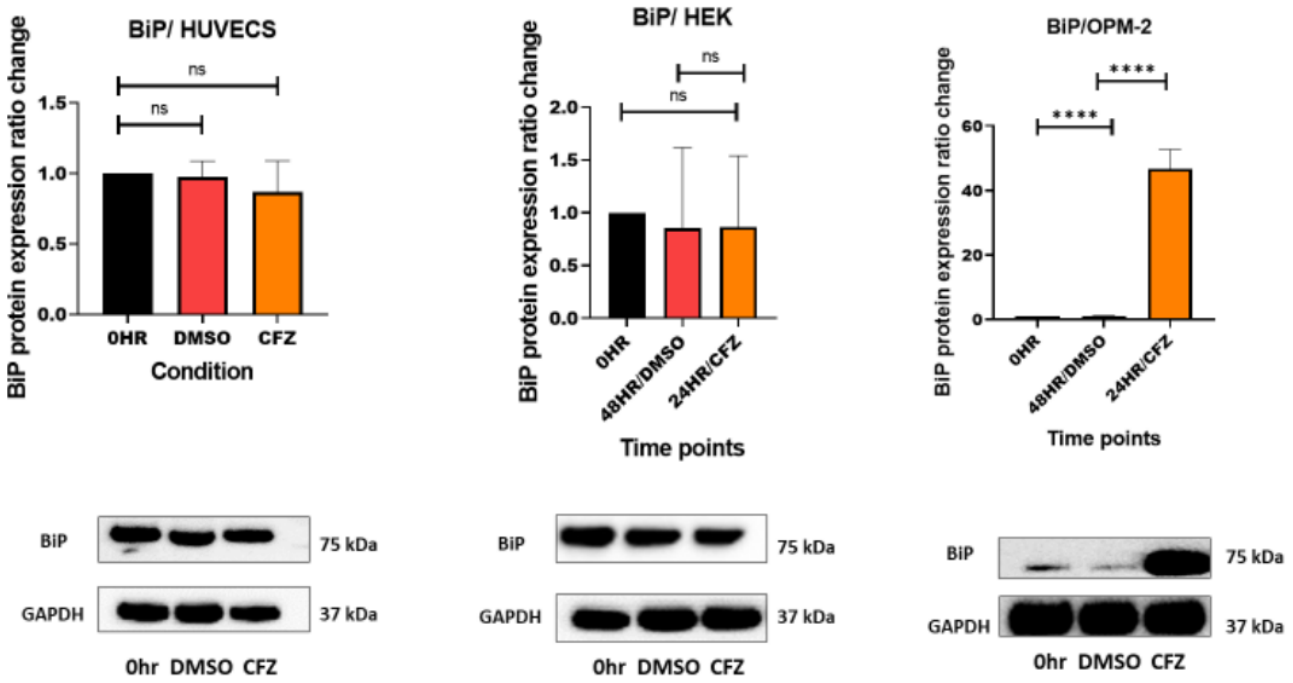
Figure 14: CFZ did not trigger apoptosis in 60% or 100% confluence HUVECs. HUVECs were cultured in two different confluences, 60% and 100%, followed the cells were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in fresh EBM-2 medium. Cells were harvested to extract protein at 0hr and 24hr. Annexin-V immune staining for 60% and 100% confluence cells. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *P < 0.05, **P < 0.01, ***P < 0.001

3.2.10 CFZ did not induce ER stress in Human embryonic kidney 293 (HEK293) cells but did induce ER stress in multiple myeloma cells (OPM-2)

According to the data obtained in this chapter, there was lacking evidence to support the hypothesis that CFZ induces ER stress in HUVECs. We hypothesised that CFZ could induce ER stress in cancer cells since they are rapidly growing and more protein-demanding cells (15), but not in normal cells. To confirm that, the HEK293 cells line, a well-known regular cell line, was selected as a negative control and the OPM-2 cell line as a positive control to examine the CFZ effect. A 100% confluence cells from each cell line were treated with 750nM CFZ and DMSO for 1hr, followed by washing the cells and resuspend them in fresh medium and left them to recover for 24hr. Interestingly, consistent with our HUVECs findings, the data obtained showed that CFZ could not induce ER stress in HEK293 cells, though there was a morphological change in CFZ treated HEK cells after 24hr recovery (**Figure. 15, A&B**). However, there was a significant induction of the ER stress in the OPM-2 cells, consistent with what has been published in the literature (16). Taken all together, indicating that CFZ is inducing ER stress in high protein demanding cells such as non-fully confluence HUVECs and OPM-2 cells, but not with normal cells that have a regular protein turnover, such as normal endothelium and HEK293 cells.


24HR
0HR
DMSO
CFZ

B


BiP/ HUVECS
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Time points
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BiP protein expression ratio change
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Figure 15: CFZ did not induce ER stress in HEK293 cells but induced ER stress in OPM-2 cells. HEK293 and OPM-2 cells were cultured up to 100% confluency, followed the cells were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS resuspended them in fresh medium. Cells were harvested to extract protein at 0hr and 24hr. ***A)*** Morphological observation of HEK293 cells using a light microscope (10X lens). ***B)*** Western Blots analysis of BiP proteins level expression and Density quantification of western blots. DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of ubiquitin between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.00$

3.2.11 CFZ is not inducing de novo proteasome synthesis in HUVECs

Our data indicated a significant induction of ubiquitination expression but no ER stress-induced in the endothelium caused by CFZ. Therefore, we asked if HUVECs recruit an alternative mechanism to avoid the induction of ER stress. Therefore, de novo proteasome synthesis is alternative cellular machinery used by the cells to overcome the harmful impact of proteasome inhibition(17). In this process, the cells produce more proteasome subunits to expand the proteasome expression for the misfolded proteins, thus preventing the lethal consequences of prolonged ER stress activation(17). In order to investigate that, the cells were treated with 750nM CFZ, followed by resuspending the cells with fresh EBM-2 medium and left them to recover for 24 hr. Both western blot and qPCR data showed that there was no induction of de novo proteasome synthesis. This was revealed by the relative expression of PS20 in all treated and no treated conditions (Figure**. 16A&B**), implying that the endothelium did not utilise this mechanism to avoid the induction of ER stress by CFZ.

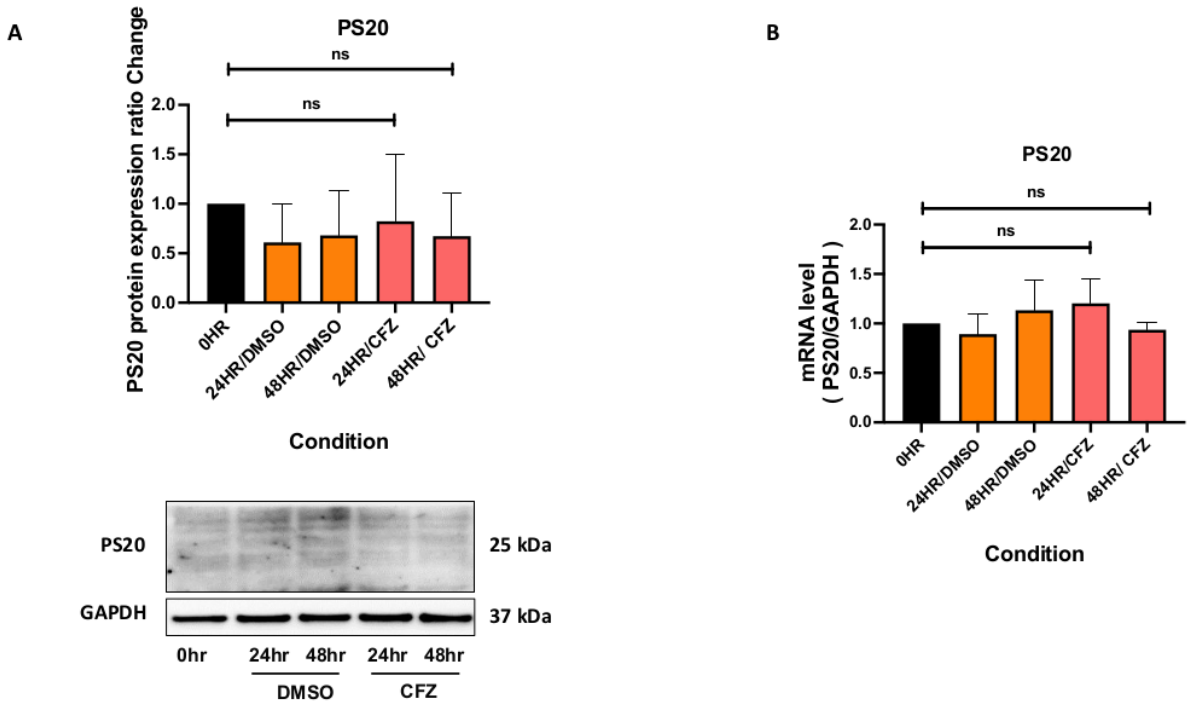

Figure 16: CFZ did not induce de novo proteasome synthesis in HUVECs. HUVECs were treated with 750nM of CFZ and DMSO for 1 hour, followed by washing the cells with PBS and resuspended them in a fresh EBM-2 medium. Cells were harvested to extract protein at 0hr and 24hr. Western blot was used to determine the extent of PS20 expression. **A)** Western Blots analysis of BiP-protein level expression and density quantification of western blot. **B)** The expression level of mRNA of PS20 in CFZ treated HUVECs and controls DMSO; Dimethyl sulfoxide, CFZ; carfilzomib. Three

technical repeats were performed in one experiment, and the quantification of data was normalized to GAPDH levels and presented as the mean ± SEM. The data represented the average of three independent experiments. The expression trends of PS20 between CFZ and DMSO cells were compared using one-way ANOVA. Significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

3.3 Discussion

Since CFZ induces ER stress in MMCs, it was initially hypothesized that similar effects would be seen in HUVECs, which is not addressed yet. The endothelial cells are essential regulators of haemostasis, permeability, and immunity; hence, examining the effects of CFZ on the endothelium is of crucial meaning. *Therefore,* ***this chapter aimed to investigate how CFZ may impact the endothelium by focusing on whether CFZ could induce ER stress in the endothelial cells***. HUVECS were since they were considered a standard endothelial model, relatively easy to grow compared to other endothelial cells, easy to obtain and economical. (18). HUVECS were treated with a single or double 750nM dose of CFZ or with different concentrations of CFZ (750nM, 1500nM, and 3000nM) for 1hr, mimicking the estimated clinical exposure average drug half-life (19). Cells were visualised, harvested, and cell lysate and genomic materials were collected for further experiments as described **(Materials & Methods).**

In this chapter, two main findings were observed in CFZ treated HUVECs. **1)** treating the cells with single/two and/or uni-concentration / multi-concentration of CFZ has not been able to induce ER stress or apoptosis in HUVECs, though there was remarkable progress morphological alteration in the drug-treated cells among all the experiments. **2)** treating **non fully** confluent HUVECs with 750nM of CFZ was adequate to induce a significant induction of ER stress. However, this was not mimicking the physiological condition since the cells form a confluent luminal monolayer structure in the human vascular system.

Initially, to investigate our hypothesis, we first examined the core functional ability of CFZ to inhibit the proteasome function in our selected cell model. This was achieved by comparing the level of ubiquitination expression between CFZ treated cells and untreated and DMSO controls. Ubiquitin is involved in ERAD mechanisms by identifying misfolded proteins for proteasomal degradation(20). Effective inhibition of the proteasome causes prolonged proteotoxic endoplasmic reticulum stress, leading

to cell death(20). Our data showed an almost three-fold increase of the ubiquitination level in CFZ treated HUVECs compared to controls at 24 hours only. This increased expression of ubiquitin at 24 hr indicates the drug's ability to induce P20 proteasome inhibition in HUVECs, which results in the accumulation of the misfolded protein in the ER lumen, subsequently activation of the ER stress. However, the decreased expression of ubiquitin at 48hr indicates rapid recovery of proteasome activity. Interruption of ER homeostasis due to a build-up of misfolded proteins can lead to ER stress(21). To determine if CFZ could induce ER stress in HUVECS, the expression of a panel of ER stress markers was investigated. Interestingly, the western blot and q-PCR data showed that CFZ was not inducing ER stress in HUVECSs at 24hr and 48 hr recovery after 1 hr treatment with CFZ. Moreover, exposure of accumulative dosage of CFZ in two consecutive days showed an enhancing of the proteasome inhibitory effect. The ubiquitination expression was progressively up-regulated, reaching the peak after 48 hr, which was not found in the single dosage experiment. However, this effect was not adequate to induce ER stress in the endothelium since there was no induction of any of the ER stress markers were examined in the CFZ accumulative experiment. In consistence, our data showed that a serial increased concentration up to 3000nM of CFZ was not achieving the activation of the ER stress in the endothelium as well. To confirm that our assays were sensitive to detect ER stress, assays were repeated following the treatment of HUVECS with tunicamycin, a known ER stress inducer(22). Tunicamycin is an antibiotic that inhibits the attachment of pre-cursor sugars structure to newly synthesized proteins within the ER resulting in the accumulation of misfolded proteins and ER stress(22). Consistent with previous observations, treatment of HUVECs with tunicamycin induced upregulation of ER stress markers; however, no increase was observed with CFZ treated cells. This data indicates that under the conditions of the assays used, CFZ cannot induce ER stress in HUVECS (22). There is various hypothesis could explain the reason behind these outcomes. **Firstly**, It is widely accepted that cancer cells have a sensitive dependence on the degradative feature of protein homeostasis because of their fast cell cycle, hyperactive protein synthesis, and several genomic mutations, which often result in the creation of defective proteins and imbalance in protein levels (22). Therefore, the high protein demand of cancerous cells, unlike the normal cells (HUVECs), combined with the

disruption of protein production that results from proteasome inhibition by CFZ, could result in fatal ER stress, which seems improbable to occur in HUVECs(23).

Secondly, when CFZ impairs ERAD which is considered one of the central cellular processes to control normal protein production and turnover, HUVECs could utilise and activate other mechanisms to degrade the unwanted proteins, such as autophagy(19). This mechanism is considered the complementary proteocatabolic method to compensate for proteasome inefficiency (19). Terminally or misfolded non-functional cytosolic proteins that are not rapidly destroyed by the UPS are susceptible to form toxic and proteolysis-refractory masses(24). These are moved in a microtubule-dependent pattern to the centrosome, forming aggresomes, selectively immersed and transported to the lysosome by the autophagic machinery(24). **Thirdly**, the load-versus-capacity model predicts that cells can avoid the harmful impacts of proteasome inhibition by attenuating the protein load in the cytosol (25). This could be another mechanism utilized by the endothelium to escape from the effect of the proteasome inhibition caused by CFZ (25). **Lastly**, it was proved that the rapid clearance of CFZ from systemic circulation < 20 min after intravenous bolus administration of CFZ by mainly extrahepatic mechanisms, epoxide hydrolase, and peptidase metabolization of CFZ(19). Therefore, the experiment time exposure (1hr) could not be adequate to induce ER stress in HUVECS. However, prolonged exposure of the cells to the treatment is not physiologically relevant, since it is well known that the drug half-life in MM patients is range between 30min to 60min(26).

Interestingly, I found that administration of physiologically relevant CFZ dosage was established to cause noticeable morphological changes in HUVECs nevertheless of studies conditions. A "cobblestone" like appearance is well-known, describing the shape of fully confluence endothelial cells (monolayer structure) when cultured in vitro(27). This shape pattern was shown in untreated, and DMSO treated HUVECs; however, in the case of CFZ treated HUVECs, progress to "stretched" spindle shape was developed through the assays. Several pieces of literature reported a similar morphological appearance when the endothelium was activated. According to Stroka et al., the endothelial cells become more elongated and stretched in response to TNF-α stimulation(28). Activated ECs showed a structural

rearrangement, including significant reorganization of the cytoskeleton F-actin and aligns alongside the central axis of the cells, which is linked to enhanced cell stiffness and contractility(29). Another author claimed a functional link between cytoskeleton dynamic and ECs morphology during activation response of HUVECs (30). There was a significant alteration in the F-actin organization of HUVECs once TNF activated the cells-α(31). In general, the morphological change was observed in this chapter indicates a perceptible effect of CFZ on HUVECs, such as structural rearrangement and EC activation, which was investigated in the next chapter.

Since all the previous experimental assays failed to reject the null hypothesis and based on the remarkable morphological alterations were observed in CFZ treated HUVECs among all the earlier experiments. Therefore, we established a nine-time points recovery assay aiming to detect any potential earlier ER stress induction that could happen and was not detected at the former selected recovery time points (24hr and 48hr). The data obtained from this assay indicated a time-dependent increase of ubiquitinated proteins and ER-stress transducers, PERK, and IRE1α, and a progressive morphological change within 24hrs after the original CFZ treatment in HUVECs. However, these inductions were not last permanently, were all these markers were returned to the baseline within 48 hr. Interestingly, CFZ was established to stimulate a significant upregulation of BiP protein expression initiated at 2hr and last up to 48 hr recovery post initial one h Carfilzomib treatment in HUVECs. Thus, the upregulation in ubiquitinated protein levels and elevated ER-stress transducers imply ER-stress is achievable in HUVECs within one CFZ treatment. However, this finding flagged conflicting data; this was about the BiP protein expression at 24 hours and 48hr in this assay and the previous experiments. While the former experiments indicated no induction of ER stress after 24 hr and 48 hr recovery, the nine-time point assay showed a lasting induction of ER stress in the endothelium after 1 hr CFZ treatment. The backward systemic root analysis of our experiments indicated that various factors could cause the conflicting outcome. Therefore, a series of experiments were conducted to resolve the raised incident. The conclusion obtained from these assays indicated that the conflicting outcome was caused by the variety of HUVECs confluence at the initiation of all experiments. Our data showed that the CFZ could not induce ER stress when the cells were fully confluence at the

beginning of the experiment. However, this was not the case in fewer confluence cells, with significant induction of ER stress. However, the latter finding does not precisely reflect the human endothelium's monolayer structure; thus, conducting the further investigation was not essentially required. ECs cover the entire internal surface of the blood vessels and perform a vital role in preserving the operational integrity of the vascular wall(32). The endothelium forms a confluent luminal monolayer structure of tightly junctional endothelial cells(32). There was an oscillatory increased trend in the protein concentration overtime during the monitoring of the cell division model(33). It is well known that endothelial cells at the growth phase require more different proteins to cope with the different requirements of cellular growth(34). Usually, growing HUVECs duplicate every 1-2days, making them in a highly active and proliferative stage (non-confluence cells)(34). However, HUVECs are less demandable of protein production when the cells form a confluent monolayer and reach the rest phase with very low turnover, where their capacity to migrate, proliferate, or invade in reaction to angiogenic growth factors will begin to reduce(35). Thus, it could be that the active growing cells are more delicate to the effect of the proteasome inhibitors, which could explain the potent induction of ER stress in the 60 % confluence HUVECs but not in the full confluence cells. Even though there was a significant induction of ER stress in the 60% confluence HUVECs, there was no induction of apoptosis in neither these cells nor the full confluence HUVECs. Prolonged disturbance of ER homeostasis leads to the activation of the unfolded protein response, a set of pathways that monitor diminishing of the ERs protein-folding function and observe the ERs conditions(36). The activation of this pathway leads to resolving the ER stress and restoring normal homeostasis by involving three significant actions(37). These are expanding the expression of molecular chaperones, suppress protein translation, and degrading misfolded proteins(37). These actions are consequences of the activation of three principal ER stress transducers ATF6, PERK, and IRE1, were all of them depend on the BiP/GRP78, a vital ER chaperone, to identify misfolded proteins(38). The downstream signalling of these transducers aims to re-establish cellular protein homeostasis and to resolve the ER stress(39). This is accomplished by expanding folding capacity, improving the ER's clearance capacity by ERAD and autophagy, and reducing folding load(39). All these

together serve as a cellular protective mechanism to defence against the harmful impact of ER stress. Thus, this could explain why there was no activation of the apoptosis in the 60 % confluence HUVECs when there was a significant induction of ER stress in the cells.

Lastly, in this chapter, I hypothesised that a similar CFZ effect would be found in normal cells but not cancer cells since they are rapidly growing cells and more suitable to the harmful impact of proteasome inhibition. Therefore, I stimulated a normal cell (HEK293) and cancer cell (OPM-2) with the same single dose concentration used in the HUVECs experiment. Interestingly, the data obtained supported and confirmed my latest hypothesis. I found a similar expression of BiP protein in HEK cells, indicating the absence of ER stress activation in these cells. On the other hand, there was a significant induction of BiP protein expression in the OPM-2 cells, indicating an activation of the ER stress in the cancer cells. Our finding was in agreement with several published pieces of literature. Yuko Mishima et al. reported an accumulation of protein aggregates and ubiquitinated proteins in the cytoplasm of MMCs that led to significant induction of ER stress and eventually to cellular death(40). Several clinical trials indicated that CFZ had a potent, selective anti-cancer effect against multiple myeloma cancer cells(41). CFZ was proved to induce a permanent inhibition to the β5 subunit of the 20S proteasome in MMCs, which led to ER stress activation (15). Carfilzomib was found to led to upregulation of BiP expression and reduced AMPKα phosphorylation in MMCs (42). According to Lisa Vincenz et al., MMCs are highly susceptible to ER stress-induced apoptosis triggered by the proteasome inhibition caused by CFZ.

In conclusion, the result of this chapter leading us to reject our initial hypothesis that CFZ induce ER stress activation in the endothelium. Alternative mechanisms, such as endothelium activation alteration of endothelium haemostasis, were hypothesised secondly and were investigated in the second chapter.

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